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PROCEEDINGS OF

THE ROYAL SOCIETY.

Section B —Biological Sciences.

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On Certain Correction Terms Required in the Equations for the Kinetics of Simple Hæmolysis

By Eric Ponder, from Washington Square College New York University

(Communicated by Sir Edward Sharpey-Schafer, F R S --Received May 21, 1931 —Revised Oct 17 1931)

1 *An Improved Method for Measuring Percentage Hæmolysis*

The most satisfactory method hitherto available for the measurement of percentage hæmolysis has been the potassium cell method described in an earlier paper (Ponder and Yeager, 1930) This method, however, has certain limitations and disadvantages, two of which are particularly conspicuous

(i) The galvanometer which records the photoelectric current takes several seconds to deflect, and several seconds more to settle at zero This determines that no more than four readings of the degree of hæmolysis can be made in a minute, and that only a few points can be obtained on the percentage hæmolysis curve for rapidly hæmolysing systems Even these points, moreover, are apt to be inaccurate, for lysis is going on during the time taken for the galvanometer to move , the degree of lysis recorded is thus always greater than that really present at the moment the photoelectric cell first receives the light The effect is to move the percentage hæmolysis curve over to the right by a distance corresponding to about 0 05 minutes , this shift is of little consequence if lysis is slow, but may introduce considerable error if it is rapid.

(ii) The method is unreliable in the sense that its efficiency depends on factors which are controlled with difficulty and which are liable to vary Photoelectric fatigue, although usually absent, may appear during any experiment, the voltage of the high tension battery or of the accumulator which supplies the lamp may vary, or the potassium cell may glow as a result of a careless exposure to the light in the interval before the cells are added to the lysin in the chamber Experience shows that these accidents often happen, and the necessity of being continually on one's guard against them makes the measurement of percentage hæmolysis much more difficult than would appear at first sight

Within the past 6 months I have found a virtually perfect method for measuring percentage hæmolysis, and as a result have discontinued using photoelectric cells This method employs as a photometer the "Stuphenphotometer" recently designed by Pulfrich of Carl Zeiss A special lamp supplies two equal beams of light, one of which passes through a light filter made by enclosing a piece of pink paper between two sheets of glass, and the other of which passes through a small glass chamber, 3 cm high, 2 cm wide and 0·5 cm thick, in which the hæmolytic system is contained The apparatus is so arranged that all the light entering the photometer must have passed either through the hæmolytic system or through the light filter The chambers themselves are surrounded by a large water jacket, electrically heated and thermostatically controlled, while the hæmolytic system is stirred by a flat paddle about 3 mm wide, situated at one side of the chamber, and driven by an electric motor

The two beams of light, one of which has traversed the hæmolytic system and the other of which has passed through the pink filter, now enter the two apertures of the photometer, which is designed so that these apertures can be reduced by turning two graduated screw heads The beams are then brought side by side so that each illuminates one-half of a split field, viewed through the Stuphenphotometer eyepiece In order to measure the intensity of one beam with respect to the other, the screw head on the side of the less bright beam is set at 100, and the other screw head is turned until the two half-fields are matched, the reading of this screw head then gives the intensity of the less bright beam as a percentage of that of the brighter The instrument has a very large range, and two intensities differing by a thousand-fold can be matched quite easily

Before using it to measure percentage hæmolysis, the apparatus requires to be calibrated. A series of standards representing 0 per cent, 10 per cent.,

. . , 100 per cent. hæmolysis is prepared by adding to 4 c.c. of saline 1·0 c.c., 0·9 c.c., . . . , 0·0 c.c. of the standard cell suspension to be used in the experiments, and thereafter 0·0 c.c., 0·1 c.c., . . . , 1·0 c.c. of the same suspension laked by freezing and thawing. About 2 c.c. of each of the standards is placed in the water-jacketed chamber, and a reading corresponding to each obtained, the screw head corresponding to the permanent light filter being fixed at some convenient position, say 50 or 80 on the scale. From the readings, a calibration curve can be drawn.

In carrying out an experiment in which a percentage hæmolysis curve is to be obtained, 0·8 c.c. of the lysin and 0·8 c.c. of saline are delivered into the chamber and allowed to come to the temperature of the water bath. The screw head corresponding to the chamber is set at the position for 0 per cent. hæmolysis, the head corresponding to the light filter being fixed at the same point as during calibration. A green filter should be used in the eyepiece, this colour being roughly complementary to the red of hæmoglobin. At a given instant 0·4 c.c. of standard suspension, warmed to the desired temperature, is added to the lysin. The contents of the chamber become rapidly mixed by the stirrer. Without delay, the observer looks into the photometer eyepiece, and verifies the reading for 0 per cent. lysis; if the two sides of the field do not match, something is obviously wrong either with the zero reading obtained on calibration or with the hæmolytic system occupying the chamber at the moment. If the zero is found correct (as it almost always is), readings of the photometer are made every 5, 10, 15, 30 or 60 seconds as desired, the observer looking continuously into the eyepiece and keeping the fields matched, and the reading on the screw head being noted at each time interval by an assistant. It is so easy to keep the half-fields of the photometer matched that readings taken at any interval represents the percentage hæmolysis present at that very instant, and not, as in the case of the potassium cell method, at an instant about 3 seconds later.

Under certain circumstances some of the standards used for calibrating the apparatus may show the "variable light transmission" reported by Kesten and Zucker (1928). The variation takes the form of a decrease in light transmission, and may amount to from 5 to 10 scale divisions of the photometer; it occurs in standards representing 0 per cent., 10 per cent., and 20 per cent. lysis, slightly in standards representing 30 per cent., and not at all in suspensions showing greater lysis. The extent of the variation and its rate of appearance depend largely on the efficiency with which the suspension is stirred, but the spontaneous change may eventually occur (after 15 to 20 minutes) even when stirring is rapid No change in light transmission takes place, however, up to 5 or 10 minutes from the time the standard is first prepared, and so such freshly prepared standards are used for calibration throughout Once the zero corresponding to 0 per cent lysis is established, it is a simple matter to make sure that the calibration is correct for any one experiment, for the reading taken immediately after the addition of the cells to the lysin in the chamber should always be that for 0 per cent lysis The effects of variable light transmission can thus be eliminated by using freshly prepared standards, arranging for adequate stirring, and checking the zero for each percentage hæmolysis curve

2 *Correction Terms for Velocity of Mixing*

The first use to which any improved method of measuring percentage hæmolysis should be put is that of checking the existing equations for the kinetics of simple hæmolytic reactions, in order to see if the existing theory continues to be adequate as the methods of measurement become more precise

One way in which calculated and experimental results can be compared has already been described at considerable length (Ponder and Yeager, 1930), but in certain respects the process may be shortened The theory states that the resistance distribution is a function of x, the quantity of lysin transformed in reacting with the cells, and not of c, the concentration of lysin used to bring about the hæmolysis It therefore follows that if we plot a series of percentage hæmolysis curves for a particular hæmolytic system and draw lines parallel to the abscissa, these lines will cut the curves at points corresponding to times at which equal quantities of lysin have been transformed, or, alternatively, if we mark off on the curves points at which equal amounts of lysin have been transformed, these points will all lie on a straight line parallel to the abscissa (*e g*, one of the dotted lines in fig 1) Lines joining points of equal lysin transformation I call isometathetic lines, the theory accordingly demands that all such lines shall satisfy the equation

$$dP/dt = 0, \qquad (1)$$

where P is the percentage hæmolysis and t the time

I have carried out a large number of experiments in which a time dilution curve and as many percentage hæmolysis curves as possible were obtained for many different hæmolytic systems. In plotting the time-dilution curves, readings were made for times from as short as 0·1 minute up to times as long as 400 or 500 minutes, the majority of the experiments being carried out at 25° The Stuphenphotometer was used for the percentage hæmolysis curves, and it was usually possible to obtain from 5 to 7 percentage hæmolysis curves for each system. The experimental data are analysed in the following way The asymptote of the time-dilution curve is first found, this gives the value of x_{100}, corresponding to 100 per cent lysis Next we find the order of the fundamental reaction, by using the process described in the previous paper last referred to The time taken for the transformation of 1, 2, 3, , units of lysin is then calculated for each value of c for which there is a percentage hæmolysis curve, the calculation of the time for successive units being continued until the time is reached for the transformation of that number of units which correspond to 100 per cent lysis On each percentage hæmolysis curve a point corresponding to the transformation of 1 unit of lysin is now marked, the points are joined, and an isometathetic line obtained. Points are next

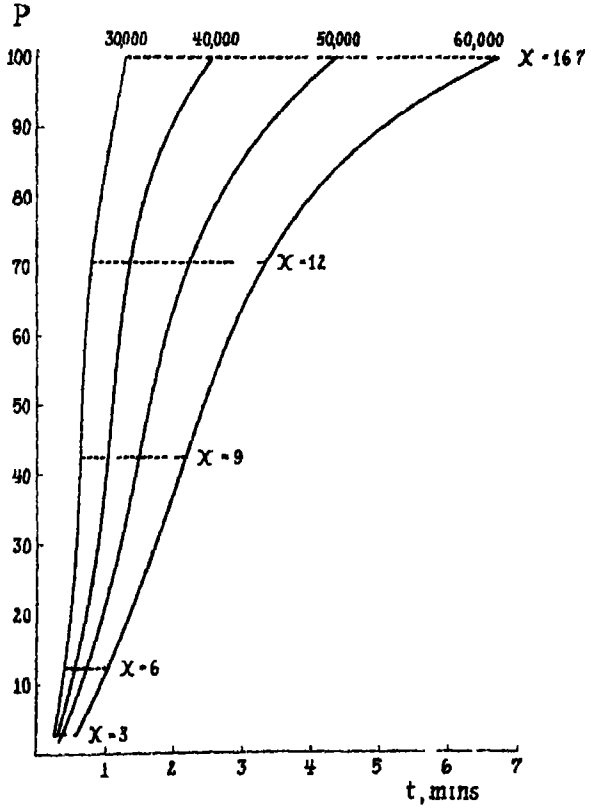

FIG 1 —Ideal isometathetic lines The figures at the top of each curve show the dilution of lysin employed, the figures opposite each line show the quantity of x (in microgrammes) transformed

marked for the transformation of 2 units, 3 units, and so on The result of joining these is a series of isometathetic lines (see figs 1 and 2)

A typical result of this procedure is shown in fig 2, where it will be seen that, contrary to expectation and to previous findings, many of the isometathetic lines are not parallel to the abscissa, but are curved in such a way as to be concave to it With saponin, digitonin, and sodium taurocholate this kind of result is invariable, and we therefore require to consider why such a divergence between experiment and theory arises

The quickest way of showing the probable cause of the curvature of the isometathetic lines is to consider an actual example, which, for convenience, is that of which fig 2 is the graphical representation

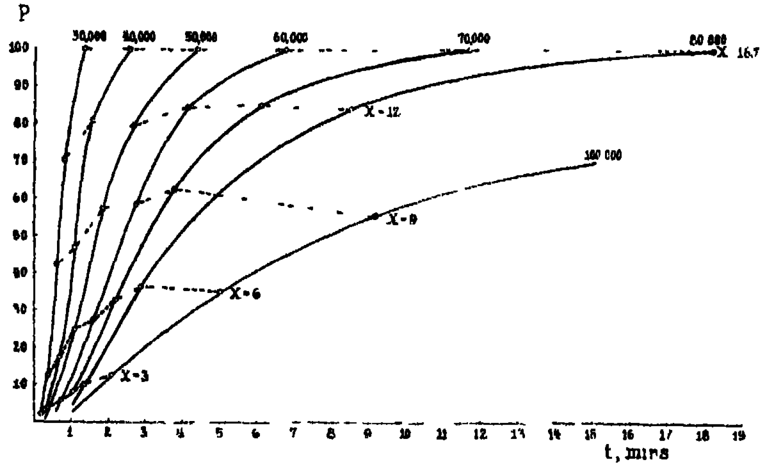

FIG 2 —Isometathetic lines observed in an experiment with human red cells and saponin For description and discussion see text Lettering has the same meaning as in Fig 1

The time-dilution curve (25° C.) was as follows —

δ	t_{obs}	t_{calc}	δ	t_{obs}	t_{calc}
10,000	0 19	0 15	60,000	6 9	6 7
20,000	0 62	0 51	70,000	12 0	11 7
30,000	1 45	1 31	80,000	22 0	18 1
40,000	2 7	2 5	100,000	53 0	53 0
50,000	4 4	4 4			

Taking c_∞ or x_{100} as 16 7 microgrammes, we find the best value of n to be 1·9 Using these values in expression (2) (see below) we obtain the calculated values found in the third column of the table. These agree excellently with the observations By means of the same formula we can obtain the time required for the transformation of various quantities of lysin (3, 6, 9, 12, 15, 16·7 microgrammes) in systems with different initial lysin concentrations; these times are shown in the following table —

x (microgm.)	c (microgrammes)						
	66·7	50·0	40·0	33·3	28·6	25·0	20·0
3	0·17	0·31	0·52	0·73	1·02	1·33	2·03
6	0·39	0·70	1·10	1·60	2·20	2·93	5·00
9	0·62	1·10	1·82	2·66	3·80	5·29	9·26
12	0·87	1·60	2·68	4·05	6·02	8·47	16·6
15	1·12	2·21	3·70	5·65	8·96	13·6	32·2
16·7	1·31	2·53	4·38	6·72	11·65	18·1	52·8

Points corresponding to each of these times are now marked off on the observed percentage hæmolysis curves, and the points corresponding to $x = 3$, $x = 6$, etc joined to give the six isometathetic lines shown in fig 2 These lines (except that for 100 per cent lysis) are concave to the t-axis instead of parallel to it, and the concavity is brought about largely by the lysis in the systems containing the greater concentration of lysin being less advanced than is expected on theoretical grounds To illustrate the same point by figures, the percentage lysis P observed for each time corresponding to each value of x and of c can be entered as in the table below, each entry of which corresponds to the entries in the table immediately preceding

x (microgm.)	c (microgrammes)						
	66·7	50·0	40·0	33·3	28·6	25·0	20·0
3	2	3	6	8	9	10	10
6	12	16	24	27	32	36	36
9	43	47	55	57	60	60	60
12	74	81	83	83	83	84	84
15	90	95	94	95	95	95	96
16·7	99	99	100	100	100	99	100

If the existing theory is correct, the figures for each row should be the same, but for all rows except that for $x = 16·7$ they increase from left to right It is this steady increase which makes the isometathetic lines appear concave to the t-axis instead of parallel to it

A little consideration, however, will show that this effect would result from some factor which moves the percentage hæmolysis curves for the higher concentrations of lysin a little to the right. More specifically, it is not difficult to imagine, when red cells in saline are added to the various concentrations of lysin, that mixing is not instantaneous, and that the fundamental reaction commences, not at zero time, but a little later. Direct evidence of such a delay will be given below, but in the meantime I shall show, on the basis of the percentage hæmolysis curves alone, that such an occurrence accounts for the divergence between observed and calculated results.

Suppose that we consider percentage hæmolysis curves for any two systems, *e.g.* that for 66·7 microgrammes and that for 40·0 microgrammes, and write down (*a*) the time, t_1, theoretically required for the transformation of 3, 6, 9, 12, 16·7 microgrammes of lysin in the first system (c = 66·7 microgrammes); (*b*) the time, t_2, required for the same amounts of lysin to be transformed in the second system (c = 40·0); (*c*) the percentage hæmolysis P corresponding to t_2; (*d*) the time, t_3, required for the same amount of lysis in the first system; and (*e*) the difference $t_3 - t_1$.

x	t_1	t_2	P	t_3	$t_3 - t_1$
3	0·17	0·52	7	0·30	0·13
6	0·38	1·1	24	0·5	0·12
9	0·62	1·82	57	0·76	0·14
12	0·87	2·68	80	1·00	0·13
16·7	1·31	4·38	100	1·45	0·14

The difference shown in the last column is virtually constant; the discrepancy noted between the two curves can therefore be wholly accounted for by assuming that a difference in the velocity of mixing delays the lysis in the first system by 0·13 minute, when compared with the second. A similar analysis of other pairs of curves leads to the same kind of conclusion.

In addition to the evidence which results from such an analysis, indications can be obtained by direct experiment that corrections for the velocity of mixing are required. The delay in complete mixing of the components of a hæmolytic system such as that generally used is probably due to the fact that 0·4 c.c. of cells in saline, a comparatively large volume, is added to 1·6 c.c. of the lysin; we may therefore investigate the effect of adding the same number of cells, suspended in smaller volumes of saline, to the same concentrations of lysin, in order to see if the time for complete lysis is reduced when the volume of added suspension becomes smaller. To do this a series of red cell suspensions are prepared, one of standard strength (S), one twice the standard (2S), one four times the standard (4S), and one half the standard strength (0·5S). By using these four suspensions, four hæmolytic systems can be prepared, the first made up of 1·9 c.c. of lysin (saponin) and 0·1 c.c. of 4S, the second of 1·8 c.c. of lysin and 0·2 c.c. of 2S, the third (the customary one) of 1·6 c.c. of lysin and 0·4 c.c. of S, and the fourth of 1·2 c.c. of lysin and 0·8 c.c. of 0·5S. These four systems are identical as regards cell content, but they differ in that mixing of cells and lysin will presumably be most rapid in the first, and least rapid in the last. Time-dilution curves can then be plotted for all four systems, and also a series of time-dilution curves for 50 per cent. hæmolysis. The former are obtained in the usual way, and the latter by setting the photometer at the reading for 50 per cent. lysis and taking the time for this degree of hæmolysis to be reached, as judged by the appearance of equality in the two half fields. The results appear below, the times being given in minutes and seconds.

1 in	4 S		2 S		S		0·5 S	
	50 per cent	100 per cent	50 per cent	100 per cent	50 per cent	100 per cent	50 per cent	100 per cent
	′ ″	′ ′	′ ′	′ ′	′ ″	′ ″	′ ″	′ ″
20,000	0 20	0 38	0 22	0 40	0 24	0 43	0 27	0 45
30,000	0 37	1 15	0 41	1 20	0 44	1 23	0 48	1 29
40,000	0 48	2 15	0 52	2 20	0 55	2 24	1 4	2 32
50,000	1 6	4 15	1 12	4 23	1 15	4 26	1 24	4 35

It will be observed that, as the quantity of fluid in which the cells are added becomes greater, the time for both 50 per cent. lysis and for 100 per cent. lysis becomes longer, just as we would expect it to do from the analysis of percentage hæmolysis curves. The delay, moreover, is of the right magnitude (0·1 to 0·3 minutes in these dilutions), and affects the time taken in reaching 50 per cent. lysis to about the same extent as it affects the time taken for complete lysis. This is best shown by subtracting, for each dilution, the time required for the lysis of the 2S suspension from that for the 4S suspension (giving Δ_1), the time for the standard suspension from that for 4S (giving Δ_2), and the time for the 0·5 standard suspension from that for 4S (giving Δ_3), and then tabulating the differences (in seconds) as in table on following page.

For any one dilution, the three differences for 50 per cent. lysis are virtually the same as the three differences for 100 per cent. lysis; the effect of adding the cells suspended in greater volumes of saline is thus to move the percentage

1 in	50 per cent lysis.			100 per cent lysis		
	Δ_1	Δ_2	Δ_3	Δ_1	Δ_2	Δ_3
20,000	2	4	7	2	5	7
30,000	4	7	11	5	8	14
40,000	4	7	16	5	9	17
50,000	6	9	18	8	11	20

hæmolysis curves bodily to the right by an amount which depends on the concentration of lysin used and on the volume of saline in which the added cells are suspended The effect is thus similar to that which we should anticipate on the basis of the curvature of the isometathetic lines, and the similarity supplies direct evidence that the explanation of this curvature, *i e*, delay in mixing, is a correct one Each percentage hæmolysis curve obtained in experiment requires, in fact, to be moved a short distance to the left, such a displacement has, of course, a negligible effect when the concentration of lysin and the slope of the curve are small, but has a great effect in terms of percentage lysis when the concentration and slope are great.*

3 *Inhibition Terms*

Taken in conjunction with other observations, the fact that the isometathetic lines, after allowance is made for delay in mixing, have the equation $dP/dt = 0$, indicates that the two expressions

$$dx/dt = k\,(c - x)^n, \tag{2}$$

and

$$N = N_0 \int_0^x F(x) \quad dx, \tag{3}$$

are adequate for the description of the kinetics of simple hæmolytic reactions The expression for the course of the fundamental reaction (expression (2)) contains, however, no term which takes account of the known inhibitory effect of liberated hæmoglobin on the free lysin of the system, and in this respect is defective

In 1926 I supposed that a constant quantity of pigment, liberated from a given number of cells, would render inactive a constant quantity of lysin.

---

* The short delay in mixing also accounts for the fact that the values of t obtained in experiment are so often a little greater than those required by theory, when lysis is rapid (see Ponder and Yeager, 1930)

This assumption, however, led to equations containing so many constants as to be useless in practice. A much better assumption (Ponder, 1923) is that the quantity of lysin rendered inert is a function not only of the amount of liberated pigment, but also of the lysin concentration, and so, as a first approximation, we shall re-write expression (2) in the form

$$dx/dt = k\,(c - x - y)^n, \tag{4}$$

where x is the amount of lysin used up in combining with the cells, and y the amount used up in combining with the pigment. The quantity of pigment liberated into the system at any time corresponding to the formation of any given quantity of x is

$$q = \mathrm{A}\int_0^x \mathrm{F}\,(x)\,dx, \tag{5}$$

or, very roughly

$$q = \mathrm{B}x, \tag{6}$$

the sigmoid integral of the distribution being replaced by a straight line through its extremities, since the use of expression (5) leads to insoluble equations. Then $y = \mathrm{B}cx$, for, by hypothesis, it is proportional to the quantity of pigment and to the initial lysin concentration; expression (4) then becomes

$$dx/dt = k\,[c - x\,(1 + \mathrm{B}c)]^n \tag{7}$$

We can now examine expression (7) with a view to finding what effect the introduction of the inhibition term has (*a*) on time-dilution curves, (*b*) on percentage hæmolysis curves, and (*c*) on the deduced frequency distribution for red cell resistances.

(*a*) Since c is treated as a constant in both (2) and (7), and since x is always measured in terms of c, it will be clear that by changing the value of the velocity constant the expression which contains the inhibition term can be made to give the same numerical results as the expression which does not. As the velocity constant cannot be determined independently, expression (7) can thus be used to give exactly the same calculated results as expression (2), and the former will describe time-dilution curves with precisely the same accuracy as the latter.

(*b*) For the same reason, expression (7) will describe percentage hæmolysis curves in exactly the same way as does expression (2).

(*c*) The addition of the inhibition term has, however, an important effect on the distribution of red cell resistances, for the asymptote is reached in expression (2) when $c = x$, but in expression (7) when $c = x\,[1 + \mathrm{B}c]$. Let us suppose, for example, that we have obtained, as a result of analysing percentage hæmolysis curves or of applying the "direct method" already described (Ponder, 1930), a frequency distribution of red cell resistances to saponin (fig 3) On the basis of expression (2) the units on the abscissa are units of

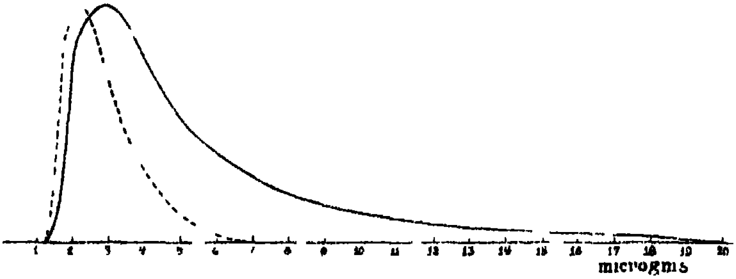

FIG 3 —Frequency Distribution of Red Cell Resistances to Saponin

c or of x, but on the basis of expression (7) they are units of c or of $x\,[1 + \mathrm{B}c]$ In the former case the curve represents the resistance distribution, but in the latter case we have to draw the new curve (dotted), whose abscissa is in units of $x = c/(1 + \mathrm{B}c)$ (In the case of the curve in the figure, B has been put equal to 0 1, a value which, as we shall see below, is quite a usual one) The result of the addition of the inhibition term, accordingly, is largely to abolish the long "tail" hitherto looked upon as characteristic of these resistance distributions, and to substitute for an unusual type of curve a type which is much more familiar

4 *The "Second Addition Effect"*

The addition of an inhibition term to the equation for the fundamental reaction enables us at once to account for a curious phenomenon which I refer to as the "second addition effect" This can be demonstrated by an experiment originally designed to measure the quantity of lysin used up in hæmolysing a given number of cells, and which may be briefly described in the following way If we produce lysis by using varying amounts of lysin c_1, c_2, c_3, etc , and if expression (2) is correct, there should be left, at the moment of complete lysis of n cells, $(c_1 - x)$, $(c_2 - x)$, $(c_3 - x)$, microgrammes of lysin These remaining quantities of lysin, however, can be made to bring about lysis of a second n cells, added at the moment of completion of the lysis of the first number, and the time which each takes to hæmolyse the second addition can be used as a measure of the quantity of lysin remaining after the lysis of the first n cells The results of such an experiment usually show that the quantity of lysin which has disappeared is far from constant, and thus point to expression (2) being erroneous. The following table, for instance, shows this quite clearly (saponin and human red cells at 25° C.):—

δ	t_1	t_2	c_1	c_2	$1{\cdot}2c_2$	Δ.	Δ/c_1
	min	min	microgm	microgm	microgm	microgm	
10,000	0·4	0·8	200	111	133	67	0·33
20,000	0·9	2·5	100	50	60	40	0·40
30,000	1·5	10·0	66·7	32	38	30	0·45
40,000	2·5	70·0	50	23	27	23	0·55
60,000	8·0		33·3	—	—	—	—
80,000	30·0		25·0		—	—	—
90,000	100·0		22·2		—		—

As judged by the time t_2 has taken to hæmolyse the second addition of cells, the amount of lysin left in the system at t_1, the completion of the lysis of the first addition, is c_2; the addition of the second quantity of cells, however, dilutes the system from 2 c.c. to 2·4 c.c., and so we need to take $1{\cdot}2c_2$ in order to get the real quantity of lysin remaining at t_1. This, subtracted from c_1, gives Δ, the quantity of lysin apparently used up in hæmolysing the first addition. This quantity is not a constant as it ought to be if expression (2) were correct, but is a function of c_1.

If expression (7) is applicable, however, the quantity of lysin used up at the end of the lysis of the first addition should be, not x, but $x(1 + Bc)$. If we assign the value 0·25 to B, we obtain the following figures:—

c_1	Δ	Δ/c_1
microgm	microgm	microgm
200	71·5	0·36
100	42·9	0·43
66·7	33·4	0·50
50	28·6	0·57

which are very similar to those usually found in experiments of this sort. The similarity is, of course, one of kind only, for the treatment of the nature of the inhibition is too approximate to lead to true quantitative agreement between experimental and calculated results; the important point, however, is that the addition of even a first approximation to an inhibition term at once accounts for a phenomenon hitherto inexplicable.

This type of result is always found experimentally when saponin or any of the glucosides are used as lysins, but an exception occurs with sodium taurocholate, which, since it is so markedly inhibited by hæmoglobin, one would expect to show the second addition effect best The effect, however, is not shown at all, for, when determined by the above method, the quantity of lysin used up in hæmolysing the first addition appears to be virtually constant —

	t_1	t_2	c_1	c_2	$1·2c_2$	Δ
1000	0·15	0·3	2000	1430	1716	284
1500	0·35	1·0	1333	869	1040	290
2000	0·75	3·0	1000	590	708	292
2500	1·2	11·0	800	433	520	280
3000	1·8	45·0	667	340	408	259
4000	6·0		500		—	
5000	20·0	-	400		-	
6000	50·0		333	—	—	-

The question therefore arises as to whether the method used for finding Δ is always applicable, and the necessity of obtaining a definite criterion of its applicability becomes all the greater when we remember that upon its accuracy depend all the results already published for the inhibitory action of plasma, serum, and hæmoglobin (Ponder, 1923, 1925)

The method depends on the comparison of the time-dilution curve of a system (system A), from which lysin has been removed either by an inhibitor or by being used up in producing lysis, with the time-dilution curve of a standard system (system B) As judged by equal times for the production of 100 per cent lysis, a concentration c_1 in system A is equal to a concentration c_2 in system B , the amount of lysin inhibited or otherwise removed from system B is then given by

$$\Delta = (c_1 - c_2) \qquad (8)^*$$

But this procedure is valid *only* if the difference between the two systems consists in the irreversible removal of lysin from system A, and not if the fundamental reactions or resistance distributions in the two systems are different , more specifically, if k, n, or $F(x)$ in expressions (2) or (3) are not the same in systems A and B, expression (8) will lead to incorrect results

A little consideration will show that there is only one valid test for the similarity of k, n, and $F(x)$ in the two systems, viz , that the percentage hæmolysis curve corresponding to c_1 acting in system A shall coincide with the percentage hæmolysis curve for c_2 in system B In practice, however, the complete coincidence is too difficult to demonstrate as a routine measure, and it is enough

* Or $\Delta = (c_1 - 1·2\, c_2)$ if the system is diluted by further addition of cell suspension

to show that the points for 50 per cent. and for 100 per cent. hæmolysis coincide. This is best done in the following way. Two time-dilution curves are plotted for the standard system B, the first showing the times required for various dilutions to bring about complete lysis, and the second showing the times for 50 per cent. lysis. The times required for 100 per cent. and for 50 per cent. lysis in system A are then found in a similar manner. Let c_1 be the initial lysin concentration in system A, c_2 the concentration apparently left after lysis of a first addition (or apparently active in the presence of the inhibitor) as judged by the time taken to produce 50 per cent. lysis of a second addition, and c_3, the concentration apparently left after the lysis of the first addition, as judged by the time taken for complete lysis of a second addition.

Then, if k, n, and $F(x)$ are the same in both systems,

$$\left.\begin{aligned} & & \Delta_2/\Delta_1 &= 1 \\ &\text{where} & \Delta_1 &= c_1 - 1.2c_2 \\ &\text{and} & \Delta_2 &= c_1 - 1.2c_3 \end{aligned}\right\} \qquad (9)$$

If Δ_2/Δ_1 differs substantially from unity, the particular inhibitor investigated must produce changes in the system other than the mere inactivation of lysin, and this method of analysis is unsound.

In the case of the second addition effect with saponin, Δ_2/Δ_1 is always sufficiently near unity, as the following experiment shows:—

c_1	c_2	c_3	Δ_1	Δ_2	Δ_2/Δ_1
200	95	90	86	92	1.057
167	70	67	83	87	1.048
133	56	53	66	69	1.045
100	41	40	51	52	1.019

In the case of the second addition effect with sodium taurocholate, on the other hand, the lysis of the first addition so changes the system that this method of analysis cannot be used:—

c_1	c_2	c_3	Δ_1	Δ_2	Δ_2/Δ_1
2000	1176	1333	589	400	0.679
1667	980	1075	491	377	0.767
1333	741	800	444	373	0.840
1000	526	550	369	340	0.921

The last important case is that in which serum or plasma is used as an inhibitor The results of an experiment on inhibition of saponin hæmolysis by rabbit serum are given in an abbreviated form in the following table, and the constancy of the ratio Δ_2/Δ_1 shows that the serum may be considered as having no other effect than that of inactivating the lysin irreversibly

Serum	c_1	c_2	c_3	Δ_1	Δ_2	Δ_2/Δ_1
c c						
0 04	200	125	130	75	70	0 93
	100	41	42	59	58	0 98
0 02	100	57	59	43	41	0 95
	80	43	42	37	38	1 03
0 01	100	77	76	23	24	1 04
	80	60	60	20	20	1 00
	67	53	53	14	14	1 00
0 005	80	63	64	17	16	0 94
	50	45	45	5	5	1 00

In the next table are shown results for the inhibition of sodium taurocholate lysis by serum

Serum	c_1	c_2	c_3	Δ_1	Δ_2	Δ_2/Δ_1
c c						
0 08	2000	420	430	1580	1570	0 99
0 04	2000	1170	1170	830	830	1 00
	1000	470	480	530	520	0 98
0 01	1000	530	540	470	460	0 98
	800	430	425	370	375	1 01
0 005	800	526	530	274	270	0 98
	667	434	444	233	223	0 96

The fact that Δ_2/Δ_1 is so nearly unity in all cases and in both tables shows that the phenomenon of inhibition by serum can properly be treated as the result of the inactivation of lysin, the relations between Δ_2 and c_3, described in previous papers, have therefore to be taken just as they stand They are certainly complex, and at present rather unsatisfactory, but the complexity does not depend on the erroneous nature of the supposition that the serum merely inactivates lysin

Summary

This paper is concerned principally with the effect of introducing into the equations which describe the kinetics of simple hæmolytic systems a term which takes account of the fact that the hæmoglobin liberated by the cells which first undergo lysis has an inhibitory effect on the lysis of the remainder.

It is shown that the addition of this new term does not in any way affect the numerical results previously obtained for time-dilution and percentage hæmolysis curves, the only result of its addition, so far as these curves are concerned, being a change in the value of the "velocity constant" of the equation for the fundamental reaction between cells and lysin An important difference is introduced, however, in the form of the calculated distribution of red cell resistances If this distribution is computed by the use of an expression which contains no term for the inhibition by hæmoglobin (as heretofore), it shows a very long tail stretching in the direction of the upper extreme; if it is computed by using the expression which contains the inhibition term, on the other hand, the peculiar tail disappears, and the distribution becomes one of a much more common type

By means of a new optical method for measuring percentage hæmolysis, it is shown that additional correction terms of very small magnitude require to be added to the theoretical equations in order to allow for delay which occurs in the complete mixing of the added cells with the other components of the hæmolytic systems If this delay in mixing is allowed for, the theoretical and experimental values obtained for time-dilution curves and percentage hæmolysis curves agree almost perfectly

The remainder of the paper is concerned with various aspects of the inhibition produced by hæmoglobin and the plasma, and with the peculiar results obtained when we attempt to measure the amount of lysin used up in hæmolysing a given number of cells by measuring the time taken by the remaining lysin to hæmolyse a second quantity of suspension added at the moment of complete lysis of the first number of cells These investigations do not lend themselves to summarisation, but the results are in accordance with expectation

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The Kinetics of Hæmolysis in Amboceptor-complement Systems

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This paper, which contains a description of the kinetics of hæmolysis in systems containing amboceptor and complement, is the first of a series of papers which will deal with the kinetics of lysis in various complex hæmolytic systems, *i.e*, systems in which the lysin (*e g*, complement) is not capable of acting except in the presence of a second substance, usually a "sensitising agent" (*e g*, amboceptor)

1. *Materials and Methods*

The methods which have been used in this investigation are essentially the same as those developed in connection with simpler hæmolytic systems. Individually they are best described in the sections of this paper to which they refer, but repetition will be avoided if certain general points are dealt with here

(*a*) *Cell Suspensions*—Nearly all the experiments to be described were carried out with systems containing sheep cells, anti-sheep amboceptor, and guinea-pig complement, the components being added in that order The cell suspension, made up from cells washed three times, consists of 4 c c of defibrinated sheep's blood finally suspended in 80 c c of 0 85 per cent NaCl This latter should be freshly prepared, and the suspension itself should be prepared shortly before the commencement of the experiment in which it is to be used I keep it at 25° C * rather than in the refrigerator

(*b*) *Amboceptor*—The anti-sheep amboceptor supplied by Messrs Burroughs & Welcome is used. If the anti-serum used belongs to the same batch, it ensures that the amboceptor used on any one day possesses about the same activity as that used on another, and something like constancy is introduced into one variable of the experiments The amboceptor is diluted with 0·85

* Details of this sort may seem trivial, but are quite important, for two suspensions, one kept at 25° C and the other at 3° C, may yield different results In this case the differences are numerical only and not differences of kind, but in some cases, however, equally small variations of technique may change the entire result of an experiment.

per cent NaCl shortly before use The most convenient way of preparing the dilutions is to use 50 c c flasks, in which dilutions of from 1 in 10, 1 in 20, .., 1 in 100, 1 in 5000 are made and kept at room temperature (25° C)

(c) *Complement* —The complement is obtained from etherised guinea-pigs about 18 hours before the experiments are commenced, the serum being allowed to separate in the refrigerator at 3° C An hour or two before it is required, the serum is decanted, treated for 30 minutes at 3° C with an equal volume of washed sheep cells to remove natural hæmolysins, centrifuged, and returned to the refrigerator

Preparations are now made for diluting the complement Ice-cold saline is run into a series of 50 c c flasks, and a suitable quantity of the complement-containing serum added to each so as to give a series of dilutions of complement from 1 in 20 to about 1 in 200 The flasks are kept in the refrigerator until needed If these dilutions are properly made, the complement-containing serum will have been kept at 3° C for a few minutes after the withdrawal of the blood of the guinea-pig to the time when it is about to be introduced into the hæmolytic systems Success in quantitative work depends very largely on these precautions in keeping the complement at a low temperature, under these conditions its " rate of decay " is reduced to a minimum at the time of the experiment, and it behaves like a stable hæmolysin.

(d) *The Hæmolytic Systems* —These are prepared so as to have a total volume of 2 c c , and are contained either in small glass tubes in a water bath at 37° C. or in the glass chamber of the Stuphenphotometer (Ponder, 1932) at 37° C exactly as if they were simple hæmolytic systems Although the procedure may vary in different experiments, the hæmolytic systems are typically made up in the following way In a series of tubes are placed 0 8 c c of various dilutions of amboceptor, from 1 in 10 to 1 in 5000 The tubes are then placed in the water bath A few minutes are allowed to elapse to enable them to reach the temperature of the bath, 0·4 c.c of warmed cell suspension is then added to each. Sensitisation of the cells occurs very rapidly, but it is as well to allow a measured time to elapse between the addition of the cells and the subsequent addition of the complement—a convenient time is 5 minutes While the sensitisation is proceeding, one of the flasks containing one of the dilutions of complement (say 1 in 20) is taken from the refrigerator, some of its contents transferred to a large tube, and placed in the water bath, the flask is at once returned to the refrigerator By the time the 5-minute sensitisation period has elapsed, the complement will have attained the temperature of 37° C., 0·8 c.c of it is then added to each of the tubes containing amboceptor and cells, and the hæmolytic systems are thus completed. The time for complete lysis is observed for each system. This gives t, the time for complete lysis, in systems containing varying amounts of amboceptor but a constant amount of complement. The whole procedure is then repeated with the next dilution of complement (*e.g.*, 1 in 30), and so on. In this way t can be obtained (if necessary, by interpolation) for systems containing any given dilution of amboceptor acting in conjunction with any given dilution of complement.

Since two factors, the dilution of amboceptor and the dilution of complement, are varied in these hæmolytic systems, and since, as will be seen later, the components can be added in different orders with different results,* it is necessary to have some short way of designating the way in which a hæmolytic system is made up. In this and in subsequent papers the order in which a system is made up will be denoted by the order in which the symbols for the various components are written, and the dilution of each component by a suffix, *e.g.* the system $A_{10}c\,C_{20}$ denotes that the cells c (contained in the usual volume of suspension) are added *to a* 1 *in* 10 *dilution of the amboceptor* A, the complement C being added later in a 1 in 20 dilution. The volume of diluted amboceptor and of diluted complement is usually 0·8 c.c. of each, and the volume of suspension 0·4 c.c., for, conformably with the methods used for the simple lysins, the total volume of the system is 2·0 c.c. The concentration of amboceptor and of complement, in the completed system, can then be readily calculated, *e.g.*, 0·8 c.c. of A_{10} gives $A = 0{\cdot}08$ c.c. (in arbitrary units), and 0·8 c.c. of C_{20} gives $C = 0{\cdot}04$ c.c. The system $A_{10}c\,C_{20}$ might thus be denoted $A_{0\cdot08}c\,C_{0\cdot04}$; sometimes this way of writing it is more specific.

2. *Complement Varying, Amboceptor Constant.*

If the quantity of amboceptor is kept constant, the time for complete hæmolysis increases in a regular manner as the concentration of complement is decreased. By the use of the technique described, a time-dilution curve, such as is shown in fig. 1, can be obtained by plotting t against the dilution of complement. This curve closely resembles a time-dilution curve for a typical simple hæmolysin, for it passes very nearly, if not quite, through the origin, and proceeds to an asymptote corresponding to a quantity of complement C_∞, or x, which produces complete lysis in infinite time. In order that the properties of this curve may be clearly understood, I shall use a numerical example taken from the table on p 25 (Table III), this example refers to a

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* When the order in which the components of a hæmolytic system are mixed affects the result, I call the system catastœchic, and when the order of mixing is immaterial, I refer to the system as an acatastœchic (*e.g.*, saponin-cell serum systems, which are identical with saponin-serum-cell systems).

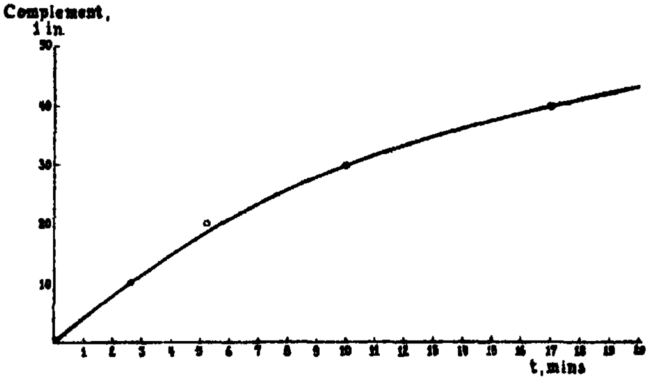

FIG 1 —Time-dilution curve for complement acting in the presence of a constant quantity of amboceptor The circles are experimental points, while the continuous line is calculated

system containing amboceptor 1 in 200 The times for complete lysis vary with the quantity of complement C in the following way —

Table I

C	0 080	0 040	0 027	0 020	0 016
(Dilution)	1 in 10	1 in 20	1 in 30	1 in 40	1 in 50
t	2 6	5 2	10	17	35

Experience shows that a curve such as this passes to its asymptote very rapidly, that is, that even a slight reduction in the complement concentration which gives complete lysis in 30–60 minutes usually results in lysis being incomplete even after 6 to 8 hours In this case we may therefore provisionally set the asymptote at $x = 0\ 015$, and, treating the curve as a typical time-dilution curve, proceed to analyse it in the usual way (Ponder and Yeager, 1930) The results of the analysis are as tabulated on the following page

The close agreement between the third and sixth columns shows that the curve may be treated as a time-dilution curve for a simple hæmolytic system in which the fundamental reaction is proceeding according to the expression

$$dx/dt = k(C - x)^n \qquad (1)$$

and in which $n = 1{\cdot}0$ approximately A slightly better fit could probably be obtained if we were to put $n = 1{\cdot}05$, for the observed ratio (fourth column)

Table II.*

P	δ	t, experimental	Ratio, experimental	Ratio, $n = 1·0$	t, calculated $n = 1·0$
12·2	6·5	1·6	15·0	14·4	1·7
24·4	13·0	3·2	7·50	6·80	3·5
36·6	19·5	5·0	4·80	4·13	5·8
48·8	26·0	7·8	3·07	2·83	8·5
61·0	32·5	11·0	2·18	1·87	12·8
73·2	39·0	16·5	1·45	1·43	16·8
85·4	45·5	24·0	1·00	1·00	24·0
97·6	52·0	50·0	0·480	0·506	47·4
100·0	53·3	∞	—	—	∞

is generally a little greater than that for $n = 1·0$, although less than that for $n = 1·1$; the fit as shown in the table, however, is sufficiently good.

But the demonstration that complement acts as a simple hæmolysin in systems containing a constant quantity of amboceptor is not completed by showing that expression (1) applies to the curve relating time for complete lysis to complement concentration; in addition it is necessary to show that the percentage hæmolysis curves are obtained by solving expression (1) simultaneously with

$$N = N_0 \int_0^x F(x)\, dx, \tag{2}$$

where $N = N_0F(x)$ expresses the resistance distribution of the cells to the lysin. The simplest way of doing this has been discussed in the paper immediately preceding (Ponder, 1932) where it is shown that a series of lines (isometathetic lines) can be drawn, each of which passes through points at which a constant amount of lysin has been transformed, through the family of percentage hæmolysis curves obtained for systems containing different amounts of lysin; if these isometathetic lines are substantially parallel to the abscissa, *i.e.*, if

$$dP/dt = 0 \tag{3}$$

in each case, one can be reasonably sure that the lysin acts as a simple lysin, and that its kinetics are adequately expressed by equations (1) and (2).

To illustrate the results of this procedure, I show in fig. 2 a series of percentage hæmolysis curves and the corresponding isometathetic lines. The observations are plotted in a somewhat simpler way than in the paper just referred to;

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* For purposes of calculation it is convenient to express the quantity of complement in cubic millimetres instead of in cubic centimetres, 0·040 then becomes 40, and so on.

instead of plotting the percentage hæmolysis curves themselves, I have plotted the Stuphophotometer reading against time, and added a subsidiary scale by means of which the former can be converted into values of percentage hæmolysis P. In this particular case, the time-dilution curve observed was as follows —

C	0 040	0 027	0 020	0 016	0 013	0 01
t obs t calc	3 3 3 4	5 0 5 2	7 5 7 5	10 10 0	13 12 8	22 20 2

The calculated values are obtained by putting $n = 1\ 0$ and $x = 0{\cdot}0067$. The values of t which correspond to $x = 0\ 001$ are then calculated for each complement concentration, the points marked off on the respective curves in the figure, and an isometathetic line drawn through them The process is repeated for $x = 0\ 002$, and a second isometathetic line obtained, and so on for $x = 0\ 003$, $0\ 004$, and $0{\cdot}005$ As will be seen from the figure, these lines are substantially parallel to the abscissa, and the demonstration that complement acts as a simple lysin in systems containing a constant quantity of amboceptor is thus completed

The results of experiments such as these lead to three additional conclusions of a less fundamental kind, namely —

1 In the case of saponin I have shown that the isometathetic lines are usually not parallel to the abscissa, but become so only after corrections have been made to allow for delay in the complete mixing of the components of the system (Ponder, 1932) In the case of amboceptor-complement systems, on the other hand, the isometathetic lines usually lie parallel to the t-axis, just as theory demands Corrections for delay in mixing are therefore unnecessary, but I think that this is due to the fact that it is difficult to obtain very rapid lysis, rather than to any essential difference between these systems and those containing saponin, etc

2 From the data in fig 2 it is a simple matter to determine the form of the resistance distribution of the cells to the lysin, *i.e.*, $N = N_0F(x)$ All the percentage hæmolysis curves in this instance yield the following corresponding values of P and x,

x	1	2	3	4	5	6	6·7
P	0	1	10	40	80	94	100

as can be seen from the position of the isometathetic lines; plotting these values against each other, and differentiating graphically, we get

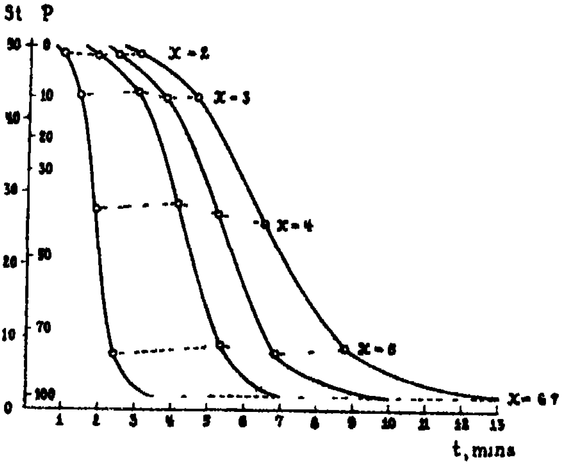

FIG 2—Isometathetic lines obtained for systems containing various amounts of complement (0 04 c c, 0 02 c c, 0 016 c c, and 0 013 c c) and a constant quantity of amboceptor. The ordinate has two scales, P representing percentage hæmolysis and St representing the Stupho reading These figures, when compared with those shown in the paper immediately preceding, will be seen to be upside down, this is because the Stupho readings, rather than the figures for percentage hæmolysis, are used for plotting the curves

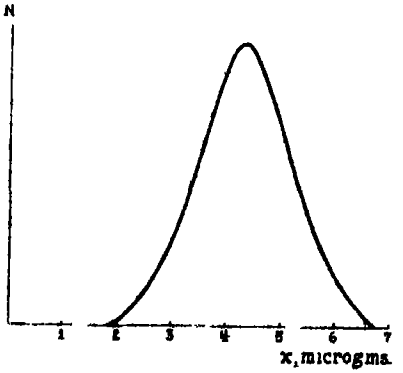

FIG 3—A typical frequency distribution of the resistance of sensitised sheep cells to the lysin complement. For description see text

the resistance distribution shown in fig 3. This distribution is characterised by its relative symmetry and the relatively large quantity of lysin which requires to be transformed before even the least resistant cell is hæmolysed

3. The distribution does not show the long tail in the direction of the upper extreme which is met with in the case of saponin In the paper immediately preceding this, the presence of the tail has been shown to be largely due to the omission, from the equation for the fundamental reaction, of a term which takes account of the inhibitory effect of liberated hæmoglobin We might, therefore, deduce from the absence of the tail in the case of complement that the complement which acts in a hæmolytic system is not inhibited by liberated hæmoglobin It can easily be shown, however, that the addition of hæmoglobin to complement, before the cells are added to the system, results in inhibition. Taken together, these facts suggest that the complement in a system in which the hæmoglobin is liberated after the mixing of complement and cells is in a different state from that in a system in which the cells are added after the mixing of complement and hæmoglobin We shall see later that this difference probably consists in the complement being absorbed by the cells in the first system, in the absorbed state it seems to suffer no inhibition when hæmoglobin is liberated subsequently.

3 *Complement and Amboceptor both varying*

When the quantity of complement used in the hæmolytic system is plotted against the time for complete lysis one obtains a different time-dilution curve for each concentration of amboceptor present, we have already seen that each such curve can be analysed in terms of three constants, *k*, *x*, and *n*, and we have now to find how these constants are affected by the quantity of amboceptor used

By means of an extensive experiment in which various dilutions of complement and of amboceptor are used as described above (Section 2), the following typical data can be compiled —

Table III

	C_{10}	C_{20}	C_{30}	C_{40}	C_{50}	C_{60}	C_{80}
A_{10}	1 4	2 5	3 8	5 2	7 7	12	20
A_{20}	1 2	2 2	3 7	5 0	7 0	10	21
A_{40}	1 2	2 3	3 6	5 3	7 6	11	24
A_{60}	1 4	2 5	4 2	6 3	9 1	14	—
A_{80}	1 5	2 9	4 8	7 8	12	19	—
A_{100}	1 8	3 5	6 1	9 2	14	27	—
A_{200}	2 6	5 2	10	17	36	—	—
A_{400}	4 7	10	25	—	—	—	—
A_{600}	7 5	17	50	—	—	—	—
A_{800}	11	28	—	—	—	—	—
A_{1000}	20	—	—	—	—	—	—
A_{2000}	60	—	—	—	—	—	—

The results for each dilution of amboceptor are to be treated as results for a distinct hæmolytic system; in this way we have the data for no less than 12 time-dilution curves shown in the above table, and may proceed to analyse each of them in the usual way. In every case $n = 1 \cdot 0$ (at least approximately enough for our purpose), and by using the following values for x and k (Table IV) we obtain the following calculated results (Table V) which should be compared with the experimental values shown in Table III.

Table IV

	A_{10}	A_{20}	A_{40}	A_{60}	A_{80}	A_{100}	A_{200}	A_{400}	A_{600}	A_{800}	A_{1000}	A_{2000}
$x \times 10^6$	—	9·0	9·2	10	11	12	15	20	25	40	—	—
$k \times 10^3$	—	11·6	11·3	10·9	10·1	9·7	8·2	6·5	5·9	5·0	—	—

Table V

	C_{10}	C_{20}	C_{30}	C_{40}	C_{50}	C_{60}	C_{80}
A_{10}	—	—	—	—	—	—	—
A_{20}	1·1	2·2	3·5	5·2	7·2	9·8	20
A_{40}	1·1	2·3	3·7	5·4	7·6	11	23
A_{60}	1·2	2·7	4·3	6·4	9·1	13	—
A_{80}	1·5	3·2	5·2	8·0	12	18	—
A_{100}	1·7	3·7	6·1	9·5	14	24	—
A_{200}	2·6	5·8	10	17	34	—	—
A_{400}	4·5	11	21	—	—	—	—
A_{600}	6·5	18	56	—	—	—	—
A_{800}	9·5	28	—	—	—	—	—
A_{1000}	—	-	—	—	—	—	—
A_{2000}	—		—	—	—	.	—

The agreement between the theoretical and the observed results is very good indeed, better, in fact, than is usually met with in experiments on simpler hæmolytic systems. This is probably associated with the value of n being unity, under which circumstances the time-dilution curves can be fitted with a minimum of trouble. It is impossible, of course, to obtain values for x and k corresponding to A_{1000} and A_{2000}, for there is only one experimental point for each, and I have not calculated the values of these constants for A_{10}, since systems containing so much amboceptor hæmolyse more slowly than those containing less, *e g.*, A_{20}. This relatively slow lysis in systems containing a very high concentration of amboceptor is constantly observed, but constitutes a departure which is of no immediate interest, since such large amounts of amboceptor would rarely or never be used in ordinary experimental work.

A similar slowing-down of lysis may be associated with very high concentrations of complement, and it is not improbable that the retardation is related to the large quantities of serum proteins which are introduced into systems containing undiluted complement or very concentrated amboceptor.

The way in which x and k vary with the quantity of amboceptor is shown in fig 4, which gives in graphical form the data contained in Table IV As the quantity of amboceptor becomes less, x, the quantity of complement required to produce complete lysis of the cells, apparently becomes greater, and tends to become infinite when the quantity of amboceptor is zero If the concentration of amboceptor becomes very great, on the other hand, x tends (in this case) to a value about 0 008, *i e*, even if the quantity of amboceptor is increased as much as possible, the quantity of complement required to bring about lysis tends to a constant minimal value The value of k, the " velocity constant," behaves in a rather similar fashion As the amboceptor concentration approaches zero, k apparently approaches zero also, but as the amboceptor

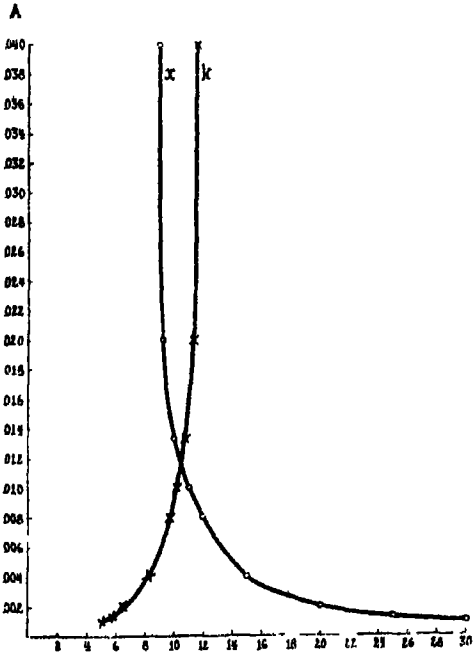

FIG 4 —Curve showing the way in which the constants x and k vary with the amount of amboceptor used for sensitising the cells The ordinate shows the quantity of amboceptor in cubic centimetres, the figures on the abscissa, when multiplied by 10^{-3}, give the values of x and k observed.

concentration is indefinitely increased, k tends to a constant value of about 0·0120

The relation between the amboceptor concentration and x, the amount of complement which has to be used to bring about lysis, is familiar in a qualitative sort of way, for it has long been known that it is possible to decrease the amount of complement in a hæmolytic system if the amount of amboceptor is increased, and still to get lysis after a long time, say, 2 or 3 hours This is often referred to as the reciprocal or interchangeable action of complement and amboceptor (see Scheller, 1910, Thiele and Embelton, 1914, and Hyde and Parsons, 1927), "a decrease in the concentration of either component being compensated for by an increase in the other" (Eagle and Brewer, 1929) Quantitatively speaking, however, this phenomenon has never been satisfactorily accounted for, although, as will be seen below, the hypothesis advanced by Eagle and Brewer contains all the essentials of the correct explanation

4 *The Absorption of Amboceptor*

As soon as we attempt to develop a hypothesis which will account for the relation, shown in fig 4, between the quantity of amboceptor A and the quantity of complement x required for complete lysis, we are confronted with a difficulty which does not arise, at least in an acute form, in problems relating to simpler hæmolytic systems The difficulty is that the properties of the hæmolytic system depend on the "degree of sensitisation" of the cells by the amboceptor, and that this may be supposed to be determined by the quantity of amboceptor A taken up by, fixed by, or absorbed by, the cells rather than by the quantity A introduced into the tube, the quantity so taken up, however, is an unknown function of A. Looked at from a purely practical standpoint, there are three possibilities, which apply equally to sensitising agent and hæmolysins themselves —

(i) The concentration A introduced into the system and the concentration which is effective in sensitising the cells (or, in general, acting on the cells) may be the same

(ii) The concentration A may be a linear function of the concentration A, *i.e*, the sensitising agent (or lysin) may be concentrated at the cell surface, but the quantity concentrated a constant fraction of the quantity introduced Cases of this kind give rise to no difficulty, for the effective concentration of lysin or sensitising agent is always a multiple of the quantity introduced, and to render time-dilution curves, etc., correct in terms of any units which may be employed, all that it is necessary to do is to multiply every figure for lysin concentration, etc , by a constant

(iii) The third possibility is that A may be a complex function of A, as, for instance, in the case of the sensitising agent, brilliant green Here the amount of dye taken up by the cells is not linear with the quantity introduced, but varies with the latter in some complex way not yet understood If the relation between A and A can be found, it may be possible to solve the problem of the kinetics of the system , if it cannot, one is working with a system in which the concentration of one component is unknown, and the kinetics cannot be completely solved If Eagle's statement were true that the quantity of complement "fixed" by sensitised cells follows the adsorption isotherm in a rather imperfect manner, then complement would fall into this third class of lysins, and the kinetics of amboceptor-complement systems would be virtually insoluble

In the case of amboceptor, it is relatively easy to establish the relation between the quantity initially introduced into the system and the quantity which, by being fixed by or absorbed by the cells, confers on them the sensitisation The method used is as follows

1 6 c c of various dilutions of amboceptor (A_{10}, A_{20}, , A_{1000}) is pipetted into a series of tubes, and 0 4 c c of standard sheep cell suspension added to each * The tubes are allowed to stand for 5 minutes at 37° C They are then centrifuged, and 0 8 c c of the supernatant fluid removed from each into another series of tubes I shall refer to this series as series 1

To each of a second series of tubes is added 1 6 c c of the various dilutions of amboceptor and 0 4 c c of saline From each tube 0 8 c c is removed and placed in another series of tubes, which I shall call series 2 The contents of the tubes of series 1 and series 2 are similar in so far as the amboceptor has been diluted equally in both, but they differ in that the amboceptor in series 1 has been exposed to the red cells contained in the added suspension, while that in series 2 has not

Each of the tubes of series 1 and series 2 are now placed in the water bath at 37° C , 0 4 c c of warmed cell suspension added to each, and sensitisation allowed to proceed for 5 minutes , at the end of this time, 0 8 c c of a 1 in 30

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* It can be shown that as much amboceptor as is required can be taken up, under the particular conditions, in less than 30 seconds

dilution of complement is added to each tube, and the time for complete lysis observed In this way we obtain two curves, one of which shows the time t_1 taken by various known quantities of amboceptor c_1, acting in the presence of 1 in 30 complement, to bring about lysis, and the other of which shows the time t_2 taken by similar amboceptor concentrations which have been in contact with cells, to produce complete lysis in the presence of the same amount of complement From the data we can find c_2, the amount of amboceptor present in the solution which has been in contact with the cells, and hence the amount Δ ($= c_1 - c_2$) removed by the cells Typical results are as follows .—

Table VI

	t_1	t_2	c_1	c_2	Δ	Δ/c_1
A_{10}	0 5	2 1	0 0800	0 0160	0 0640	0 80
A_{20}	0 8	3 6	0 0400	0 0097	0 0303	0 76
A_{40}	1 6	6 0	0 0200	0 0057	0 0143	0 72
A_{60}	2 5	10	0 0133	0 0038	0 0095	0 71
A_{80}	3 5	18	0 0100	0 0024	0 0076	0 76
A_{100}	4 5	26	0 0080	0 0019	0 0061	0 76
A_{200}	9 5	—	0 0040	-	—	—
A_{400}	23	—	0 0020	—	—	—
A_{600}	60	—	0 0013	—	—	—

The constancy of the ratio in the last column shows that the amount of amboceptor removed by the cells is a constant fraction, within the limits of experimental error, of the amount with which the cells are treated The constancy of the ratio is in some respects rather surprising, for the absorption of amboceptor has the characteristics of an irreversible reaction, and it is not easy to see how such a reaction can reach an equilibrium when a constant fraction of the amboceptor has been removed from the medium in which the cells are suspended The absorption of amboceptor, however, is not the only instance in which this kind of absorption is met with in relation to hæmolysis ; sodium taurocholate is absorbed by cells in this manner, and also (probably) sodium glycocholate and sodium oleate. It is possible that this simple relation between c_1 and Δ would be replaced by a less simple one if the experimental range were sufficiently extended, but in the systems under consideration and over the range within which experiment is possible the relative constancy of the Δ/c_1 ratio seems to be an experimental fact , the relation between A and A can, accordingly, be written

$$A = \eta A, \tag{4}$$

η having, in the above example, the value 0 75 The ordinate of fig 4 is accordingly correct in arbitrary units, and, if we wish the quantity of amboceptor actually involved in the sensitisation of the cells we have only to multiply the quantities introduced into the system by the constant η

5 *The Absorption of Complement Complement varying, Amboceptor Constant*

Just as we have examined the relation between A, the quantity of amboceptor introduced into the system, and A, the quantity absorbed by the cells, so we must determine between C, the concentration of complement introduced and C, the concentration at or near the cell surfaces

This question has been very fully considered by Eagle (1929) and by Eagle and Brewer (1929) in two recent papers These investigators have shown that sensitisation by amboceptor confers upon the red cell the property of absorbing complement, and that this absorption is a necessary preliminary to lysis Complement they regard as a lysin, which is absorbed extensively when sensitisation is produced by large quantities of amboceptor, and less extensively when small amboceptor concentrations are used The velocity of lysis is determined by the amount of complement so absorbed "With more amboceptor a greater proportion of complement is 'fixed' by the cell, with more complement, a smaller proportion, but a larger absolute amount. The result is the same more complement is absorbed, and there is a corresponding acceleration of hæmolysis" These conclusions constitute, as we shall see, an entirely adequate explanation of the facts, and, were nothing more than a qualitative explanation required, I should have little to add to them. The quantitative work on which the conclusions are based, however, is erroneous and incomplete in several important respects, and fails altogether to provide a reasonable description of the kinetics of lysis in the systems under consideration accordingly I shall subject the whole problem to an investigation independent of Eagle and Brewer's work, at the same time comparing at various points their conclusions with my own *

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* Reference should also be made to two other investigations, that of McKendrick (1911) and that of Hill and Parker (1925) I have had the opportunity of discussing the first of these with Colonel McKendrick, and he informs me that he regards his experimental results as erroneous, for heated serum is not merely complement-free, but anti-complementary So far as the mathematical treatment goes, the same fundamental objection can be raised against both investigations, viz, that neither takes account of the existence of a distribution of red cell resistances to the action of the lysin This error has complicated so many studies on hæmolysis and has been referred to so often that it requires no

The first point to be determined is the nature of the distribution of complement between cells and suspension medium The experimental methods by which this point is decided are highly complicated, and require detailed description I shall use an actual example as an illustration, describing the experiment as a series of steps

1 *Preparation.*—A standard sheep cell suspension is prepared in the usual way In a series of 50 c c flasks complement dilutions of 1 in 20, 1 in 30, 1 in 40, 1 in 50, 1 in 60, and 1 in 70 are made up in ice-cold saline, they are then kept in the refrigerator A 1 in 500 dilution of amboceptor is also required. The dilution of amboceptor must be greater than about 1 in 50, for a reason which will be mentioned below.

2 *Preliminary Titration* —To a series of six tubes are added 0 8 c c. of the amboceptor and 0 4 c c. of the cell suspension Sensitisation is allowed to take place for 5 minutes at 25° C, the tubes are then placed in a centrifuge, and the cells thrown down They are rendered sticky by the action of the amboceptor, as a result the supernatant fluid can be poured off without disturbing the cells, the remains being removed from the sides and lips of the tubes with filter paper The tubes now contain the sensitised cells of 0 4 c c. of suspension contained in a very little fluid Each is shaken to and fro, so that the sticky mass of cells is broken up, and it is then placed in the water bath at 37° C About 2 c c of each complement dilution is transferred from the refrigerator to a large tube, and these tubes are placed in the water bath; after 5 minutes the small tubes containing the cells and the tubes containing the various dilutions of complement will all have assumed the temperature of 37° C

To the first of the small tubes is added 0·8 c c. of C_{20}, to the second 0 8 c.c of C_{30}, and so on, the sixth receiving 0 8 c c of the last complement dilution prepared, C_{70} The time t for complete lysis in each tube is observed to the nearest minute, and entered in the first column of the following table

The rest of the table is filled in the following way The time t corresponding to each dilution of complement is divided into tenths, and the first six tenths

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further discussion (see Brooks, 1920, and previous papers of this series). It is easy to see how it affects Hill and Parker's hypothesis, for they recognise that their equations do not account for the well-marked S-shaped curve given by increasing complement when all other conditions are kept constant, unless they introduce elaborate assumptions. If the existence of a resistance distribution were recognised, all these would become unnecessary The agreement between observed and calculated results, of course, is not really a test of the theory, for the assumptions are introduced to that end, and are not supported by any independent experimental evidence

Table VII

	t	Tube					
		1	2	3	4	5	6
C_{20}	4	0·4	0·8	1·2	1·6	2·0	2·4
C_{30}	5	0·5	1·0	1·5	2·0	2·5	3·0
C_{40}	7	0·7	1·4	2·1	2·8	3·5	4·2
C_{50}	12	1·2	2·4	3·6	4·8	6·0	7·2
C_{60}	15	1·5	3·0	4·5	6·0	7·5	9·0
C_{70}	30	3	6	9	12	15	18

are entered in the columns headed by the numbers of the tubes The reason for this procedure is that in the next part of the experiment we shall add each dilution of complement to the sensitised cells and, instead of allowing complete lysis to take place, shall stop the reaction after one-tenth, two-tenths, , six-tenths of the time required for complete hæmolysis

We now write out the figures of Table VII in the following way (Table VIII), the figures of which are used in the next step of the experiment

Table VIII

	Off	1	2	3	4	5	6
C_{20}	2·4	2·0	1·6	1·2	0·8	0·4	0
C_{30}	3·0	2·5	2·0	1·5	1·0	0·5	0
C_{40}	4·2	3·5	2·8	2·1	1·4	0·7	0
C_{50}	7·2	6·0	4·8	3·6	2·4	1·2	0
C_{60}	9·0	7·5	6·0	4·5	3·0	1·5	0
C_{70}	18	15	12	9	6	3	0

3 *Absorption of Complement*—Six small tubes, numbered 1 to 6, and containing the sensitised cells of 0·4 c c of the suspension, are prepared as above by adding 0·4 c c of the suspension to 0·8 c c of A_{500}, centrifuging and decanting the supernatant fluid They are placed in the water bath, and with them about 10 c c of the first dilution of complement, C_{20}, in a large tube. When the complement and tubes have reached 37° C, 1·0 c c of C_{20} is added to the sixth tube, a stop-watch being started at the moment of addition 0·4 minutes later (the reader should now refer to Table VIII, reading the row corresponding to C_{20} from right to left), 1·0 c c of C_{20} is added to the fifth tube; after 0·8 minutes have elapsed, the same amount of complement is added to the fourth tube, and so on The first tube thus receives 1·0 c.c of

C_{20} 2·0 minutes after the addition of complement to the sixth After 2 4 minutes are elasped from the time of the addition of complement to tube 6, all the tubes are removed from the water bath, transferred to a centrifuge, and the cells thrown down

The centrifuge used is of a special design, for it is necessary to separate the cells from the surrounding fluid with the greatest possible rapidity The centrifuge head, carrying six tubes, are made of aluminium, is fixed to the shaft of a series-wound motor, mounted vertically The head revolves at the rate of 8000 to 12,000 r p m , depending on the load, and the motor starts and stops almost instantaneously This centrifuge will throw down all the intact cells from the systems under consideration in from 0 1 to 0 2 minutes

The supernatant fluid thus obtained from the six tubes are now decanted into six clean tubes, also numbered 1 to 6 in a corresponding manner These are placed in the refrigerator They contain the complement C_{20} after it has been in contact with sensitised cells 2·4 minutes (tube 6), 2 0 minutes (tube 5), , 0 4 minutes (tube 1), and, by titrating the concentration of complement contained in each we can find the amount of complement removed from the initial concentration C_{20} after 0·4, 0 8, , 2·4 minutes in contact with the sensitised cells

The large tube from which the complement was added to the row of small tubes is removed from the water bath when the small tubes are placed in the centrifuge, and is placed in the refrigerator It is used as a standard for the final titration , it has been warmed to 37° C for exactly the same time as has the complement added to the hæmolytic systems, and, if any " decay " has taken place as a result of the warming, etc , it will have taken place equally in the complement added to the six small tubes and in that preserved as a standard

The whole process is then repeated, using the next dilution of complement, *i e* , C_{30}, and adding it to the sensitised cells in accordance with the C_{30} row of figures in Table VIII And so on for C_{40}, C_{50}, C_{60}, and C_{70}

4 *The Final Titration* —The above procedure results in one's having six sets of six tubes each, the sets corresponding to C_{20}, C_{30}, C_{40}, C_{50}, C_{60}, C_{70}, and the six tubes of each containing the complement left after each of the first six-tenths of the time required for complete lysis by the complement added The quantity of complement in each has now to be found by titration

The large tubes containing the various standard dilutions of complement which have been warmed to 37° C and subsequently replaced in the refrigerator are returned to the water bath, and 0·8 c c. of each added to a series of tubes containing 0·8 c c of A_{100} and 0·4 c c of standard cell suspension. It is as well to use a fairly high concentration of amboceptor in these systems, in order to get moderately rapid lysis In this way we obtain a time-dilution curve for the standard complement (Table IX) —

Table IX

C_{20}	C_{30}	C_{40}	C_{50}	C_{60}	C_{70}	C_{80}
2·7	4·0	5·5	7·5	9·8	14	19

The six sets of six tubes are now removed from the refrigerator, and placed in the water bath, after their contents are warmed, 0·8 c c of each is added to a series of tubes containing 0·8 c c of A_{100} and 0·4 c c of cell suspension The time for lysis is noted in each case, as follows —

Table X

	Tube					
	1	2	3	4	5	6
C_{20}	3·2	3·2	3·3	3·6		
C_{30}	4·6	4·7	4·6	4·8		
C_{40}	6·8	7·1	6·8			
C_{50}	9·5	9·5	10			
C_{60}	13	13	14			
C_{70}	—	—				

No entries are made for C_{70}, for all the tubes corresponding to this dilution of complement hæmolyse too slowly Nor are any entries made if the tube containing the complement to be added shows colouration with hæmoglobin, for this substance inhibits lysis by unabsorbed complement. It is for this reason that we are interested in the first six-tenths of the reaction only, for in the later stages the fluid obtained after separating the cells from the complement is deeply stained with pigment During the first three- or four-tenths of the time required for complete lysis, however, no cells are destroyed (at least normally), and the complement-containing fluid can be properly titrated by these methods

Examining Table X, we see that the time taken for lysis in each row is virtually the same for tubes 1, 2 and 3 (and even tube 4 is included); this means that the quantity of complement in these tubes is the same, that is, that the same amount of complement has been removed from solution by the cells, irrespective of the stage at which the reaction is stopped. Complement, in fact, is absorbed by the cells, but the absorption is complete within the first tenth of the total time taken for lysis, and no further absorption occurs as the reaction proceeds The absorption of complement is thus a rapid process, which soon reaches an equilibrium, or at least a temporary steady state, and in this respect is very like the absorption of amboceptor

Comparing the times for lysis as shown in Table X with the standard time-dilution curve (Table IX), we can now draw up the following table, in which C_1 represents the dilution of complement present in tubes 1, 2, 3, etc , C_2 the corresponding quantity left after absorption of complement has occurred, and Δ the difference $C_1 - C_2$, or the quantity of complement removed from solution by the sensitised cells from an initial concentration of complement C_1

	C_1	1 in	C_2	Δ	Δ/C_1
C_{20}	0 040	23	0 035	0 005	0 13
C_{30}	0 027	34	0 024	0 003	0 11
C_{40}	0 020	47	0 017	0 003	0 15
C_{50}	0 016	59	0 0136	0 0024	0 15
C_{60}	0 013	68	0 0117	0 0016	0 12

Considering the nature of the experiment, the ratio Δ/C_1 is remarkably constant, about one-eighth of the complement having been removed from solution by the cells in each case. The absorption of complement is therefore not only rapidly completed, but is such that the quantity absorbed is a constant fraction of that introduced, at least in the range in which experiments of this kind can be performed, and with a departure from constancy, probably largely due to experimental error, of about ±14 per cent

This result, although it agrees with Eagle and Brewer's conclusions in a qualitative way, differs from their findings in two respects

(*a*) Eagle and Brewer describe the absorption of complement ("fixation of complement") as a process which takes some considerable time to complete. I have always found, on the other hand, that the quantity of complement absorbed is taken up very rapidly, and I have been unable to detect with certainty the taking up of quantities less than or greater than that which is absorbed at the end of the first tenth of the time required for complete hæmolysis. I realise, of course, that some short interval of time must elapse during which the complement is being absorbed from solution, but this short time, so far as my experiments show, is less than one-tenth of the time for complete lysis and usually less than a third of the "latent period" during which no lysis occurs at all

I do not think that this difference of experimental result is of immediate consequence, for even in Eagle and Brewer's experiments the absorption of complement is relatively rapid, the only difference of opinion is as how rapid it is * In the equations which have been used above (expression (1)) I have treated the absorption as virtually instantaneous, which it probably is not But this and the other expressions used in this paper are no better than first approximations, and, if it should ultimately turn out that the absorption of complement takes an appreciable time, the equations can easily be slightly modified

(*b*) The second point upon which I disagree with Eagle is in the relation between C, the concentration of complement added to the system, and Δ, the quantity absorbed by the cells This is a more important matter Eagle (1929) regards the process of "fixation of complement" as an adsorption reaction, partly because he finds the relation between the quantity of complement removed from solution, and the quantity left free to be described by the adsorption isotherm, whereas I find Δ to be a constant fraction of C He has arrived at this conclusion by studying the absorption of complement by globulin suspensions and by agglutinated bacteria, and thinks that the result is of general application Curiously enough the only data given by Eagle and Brewer for the fixation of complement by red cells show that the absorbed complement is very nearly a constant fraction of that introduced (or left free) I refer to the data given in fig 3 of their paper, from which it can be shown that the ratio Δ/C_1 is fairly constant (0·65–0 75)

I do not wish to give the impression, however, that I disagree with Eagle in considering the absorption as essentially an "adsorption" rather than a stoichiometric reaction I have already remarked on the fact that it is curious that Δ is a constant fraction of C, and on the difficulty in explaining such a relation, which also exists between A and A, in simple terms But the demonstration that a reaction is of the "adsorption" type is not dependent on showing that it follows the adsorption isotherm, indeed, the Freundlich equation neither does, nor should, apply to most biological reactions, especially when they are irreversible † A good deal of difficulty, I imagine, has been introduced by Eagle's generalisation that the kinetics of all forms of complement absorption, *e g*, by red cells, bacteria, globulin suspensions, precipitates, etc., are altogether the same in a quantitative sense *

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* Their centrifuge, however, took 1 minute to bring about the separation which the rapid centrifuge described above effects in 0 1 minute The results, accordingly, are not nearly so reliable.

† Eagle's figures show that the relation of the logarithm of the amount absorbed to the logarithm of the amount left free is far from linear, even over the very restricted range.

6 *The Absorption of Complement Amboceptor and Complement both varying*

We have seen that the amount of complement absorbed by sensitised erythrocytes is a constant fraction of that introduced into the system, *i e*

$$C = \zeta C \tag{5}$$

We have now to find how the constant ζ varies as the quantity of amboceptor is varied One might think that this could be done by repeating the procedures of the last section, using different quantities of amboceptor to sensitise the cells, as soon as this is attempted, however, we meet with a curious difficulty

One of the first steps is to add 0 4 c c of cell suspension to 0 8 c c of a given dilution of amboceptor, centrifuge after 5 minutes, decant the supernatant fluid, and then add 1 0 c c of a known dilution of complement to the cells which remain This can be done successfully if the amboceptor is fairly dilute, *e g*, A_{500} But if we use a dilution of amboceptor of 1 in 50 or 1 in 100, the cells remaining after the centrifuging hæmolyse even on the addition of isotonic saline The amboceptor, in fact, if used in this way and in relatively high concentration, is a lysin itself, therein resembling other known sensitising agents all of which are lysins if used in sufficient concentration (Ponder, 1929) Accordingly, if we wish to investigate the taking up of complement by cells sensitised with, say A_{50}, A_{100}, A_{500}, and A_{1000}, we cannot use the technique described in Section 5, but must employ the more difficult method now to be described

1 *Preparation* —The cell suspension, dilutions of amboceptor, and dilutions of complement are prepared in the usual way In the experiment which I shall describe as an illustration, the complement dilutions were C_{20}, C_{30}, C_{40}, C_{50}, C_{60}, C_{80}, and C_{100}, while the amboceptor dilutions were A_{50}, A_{100}, A_{500}, and A_{1000}

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* In the case of absorption of complement by bacteria, etc, Eagle takes the amount removed after 2 hours, by which time some sort of equilibrium has presumably been reached. One cannot necessarily compare results obtained in this way with those obtained in the early portions of a hæmolytic reaction, where the cells have been in contact with the complement for a few minutes at most, and often for fractions of a minute only

2 *Preliminary Titration*.—One dilution of complement, in this case, C_{30}, is selected for determining the relation between C and C, for the former is a constant fraction of the latter, and the constant is the same for all initial values of C, as I have just shown It is impracticable to investigate the absorption from more than one complement dilution, owing to the fact that even this limited investigation takes 4 or 5 hours where four amboceptor dilutions are used

A tube is taken, and to it is added 0 8 c c of A_{50} and 0 4 c c of suspension Sensitisation is allowed to take place at 37° C for 5 minutes, and 0 8 c c of C_{30} is then added The time for complete lysis is observed, and a table similar to Table VIII drawn up

	Off	Tube					
		1	2	3	4	5	6
A_{50}	3 0	2 5	2 0	1 5	1 0	0 5	0

The procedure is repeated with A_{100}, A_{500}, and A_{1000} It will be observed that in this method the systems contain both amboceptor and complement, in the method in Section 5 the amboceptor was removed by centrifuging and decanting

3 *Absorption of Complement*.—Six tubes are taken, and to each is added 0 8 c c of A_{50} and 0 4 c c of suspension After 5 minutes sensitisation, 0·8 c c of C_{30} is added to the first tube, 0 8 c c of C_{30} to the second 0 5 minutes later, and so on, in accordance with the table The tubes are transferred to the centrifuge at the end of 3 0 minutes, and the cells thrown down The supernatant fluids, containing the unabsorbed complement and in this case, unabsorbed amboceptor also, are transferred to clean tubes and placed in the refrigerator

4 *Preparation of Standards*.—In order to determine the amount of complement left in each of the supernatant fluids obtained in the last step, we require to prepare a series of suitable standards against which they may be titrated In preparing these, it is necessary to remember that the red cells have removed amboceptor as well as complement from solution The value of η in expression (4) has, therefore, to be found for the system, as in Section 4. A series of eight tubes is then taken, and to each is added 0·8 c c of $A_{50/(1-\eta)}$ and 0·4 c c of saline, the quantity of amboceptor is thus made the same as that contained

in the tubes used in step 3, after absorption of a fraction η of amboceptor has occurred. The tubes are then placed in the water bath, and with them a series of tubes containing about 2 c.c. of C_{20}, C_{30}, …, C_{100} taken from the flasks in the refrigerator. The first of the tubes containing amboceptor and saline receives 0·8 c.c. of C_{20}, the second 0·8 c.c. of C_{30}, and so on. The eight tubes are transferred to the refrigerator. They contain known quantities of complement in the presence of 0·8 c.c. of $A_{50/(1-\eta)}$ and 0·4 c.c. saline, and constitute a series of standards against which the supernatant fluids obtained in the previous step can be titrated, for these contain unknown quantities of complement in the presence of 0·8 c.c. of $A_{50/(1-\eta)}$ and the 0·4 c.c. of saline used for suspending the cells of the suspension.

The same procedure (as in steps 3 and 4 above) is carried out using A_{100}, A_{500}, and A_{1000}, a series of standards containing known quantities of complement being prepared for each.

5. *Final Titration.*—The two series of tubes corresponding to A_{50} are removed from the refrigerator. From each of the standard tubes containing C_{20}, C_{30}, …, etc., 1·5 c.c. is transferred to a series of clean tubes. These are placed in the water bath, and 0·3 of a warmed sensitised cell suspension is added to each. This suspension is prepared by mixing equal volumes of A_{100} and standard cell suspension, washing, and resuspending to the original volume of the suspension used. The reason for using a sensitised suspension at this stage is that the quantity of amboceptor in the complement-amboceptor mixture to which the cells are added is usually too small to give reasonably rapid lysis. The time for complete lysis of each tube is observed; this gives us a time-dilution curve for C_{20}, C_{30}, …, C_{100} acting in presence of $A_{50/(1-\eta)}$ on the cells of the sensitised suspension. The values of t which are required are:—

C_{30}	C_{40}	C_{50}	C_{60}
8·0	10·5	16	25

The contents of the six tubes containing the supernatant fluid obtained by centrifuging are now titrated by transferring 1·5 c.c. from each into a series of clean tubes, and, after warming, adding 0·3 c.c. of the sensitised suspension just described. The times for lysis are:—

Tube	1	2	3	4	5	6
t	24	25	25	26	—	—

The contents of the last two tubes showed pigmentation with hæmoglobin, and so were rejected. The first four hæmolyse, however, in the same time, *i e*, 25 minutes Referring to the standard time-dilution curve, we see that the complement dilution corresponding to 25 minutes is C_{60}, the complement introduced into the system, however, was C_{30}, so just half of it has apparently been absorbed by the cells sensitised with A_{50} Thus $\zeta = 0{\cdot}5$

The supernatant fluids obtained from the systems sensitised with A_{100}, A_{500}, and A_{1000} are titrated in an exactly similar manner, each against the corresponding series of standards In every case the contents of the tubes derived from the same series and containing the supernatant fluid will be found to hæmolyse in the same time (unless hæmoglobin is present), thus indicating the rapid removal of complement in each system The time taken for complete lysis may be quite long in the systems corresponding to A_{1000}, the times taken by the standards, however, are also longer (as the total amboceptor concentration is less), and so the quantity of complement found in the supernatant fluids turns out to be greater than in the fluids derived from systems containing the greater amboceptor concentrations Thus, the titrations being completed, we can tabulate the values of ζ found for different values of A—

A_{50}	$\zeta = 0{\cdot}500$
A_{100}	$\zeta = 0{\cdot}450$
A_{500}	$\zeta = 0{\cdot}220$
A_{1000}	$\zeta = 0{\cdot}084$

As the concentration of amboceptor used for sensitising the cells decreases, a smaller and smaller proportion of any concentration of complement is absorbed This is altogether in accordance with the conclusion of Eagle and Brewer

7 *The Relation of the Absorption of Complement to the Value of the Constants of the Time-dilution Curves*

We are now in a position to consider the way in which the absorption of complement is related to the values of x and of k in the expressions which describe the time-dilution and percentage hæmolysis curves obtained when various amounts of amboceptor are used to sensitise the cells of the systems. The typical relation between these constants and the quantity of amboceptor used is shown in fig. 4 and has been discussed already.

Now x in the expression

$$kt = \log C/C - x,$$

which is the integral of expression (1) when $n = 1$, is the quantity of lysin used up in the complete lysis of the cells of the system, and corresponds to the asymptotic concentration of the time-dilution curve for the particular system concerned, *i.e.*, x is measured in the same units as C. But we have just seen that the effective quantity of complement absorbed at the cell surface is a constant fraction of C, so that

$$\mathbf{C} = \zeta C \qquad (5)$$

and so $\mathbf{x}$, the quantity of lysin actually used up in producing lysis of the cells must be a fraction of x, *i.e.*,

$$\mathbf{x} = \zeta x \qquad (6)$$

To evaluate $\mathbf{x}$, I shall use data obtained at the same time as the values of ζ in Section 8. The following table shows these values of ζ and of x corresponding to different quantities of amboceptor, the values of x being obtained by plotting time-dilution curves for the systems containing A_{50}, A_{100}, A_{500}, and A_{1000}, respectively.

Table XI

	ζ	x	$\mathbf{x}$
A_{50}	0·500	0·008	0·0040
A_{100}	0·450	0·010	0·0045
A_{500}	0·220	0·020	0·0044
A_{1000}	0·084	0·060	0·0050

The remarkable nature of this result will be seen immediately; $\mathbf{x}$, or ζx, has a nearly constant value, *i.e.*, the quantity of lysin used up in producing complete lysis of the cells tends to be constant, irrespective of the quantity of amboceptor used for sensitisation. Complement accordingly behaves like a simple hæmolysin such as saponin, and the reason that many time-dilution curves can be obtained when the amount of amboceptor is varied is that each quantity of amboceptor determines that a different fraction of the added complement shall be absorbed. The greater the quantity of amboceptor, the greater the fraction becomes, *e.g.*, if the quantity of amboceptor is great we have a large fraction absorbed, such as 0·5, from a comparatively dilute complement, C_{80}, but if the quantity of amboceptor is small, a smaller fraction, *e.g.*, 0·25, is absorbed from the less dilute complement C_{40}. The two processes clearly give the same concentration of complement at the cell interfaces. All the time-dilution curves obtained when different quantities of amboceptor are used accordingly merge into one single time-dilution curve when the absorption of complement is taken into account, and when C is replaced by $\mathbf{C}$, and x by $\mathbf{x}$.

In like manner, the curve marked x in fig 4 becomes a straight line when the absorption of the complement is allowed for Reference to that figure will show that the curve passes up very steeply as the quantity of amboceptor increases, the straight line referred to obviously tends to be the tangent to this ascending part of the curve at some point corresponding to the maximum quantity of amboceptor which can be absorbed by the cells, and which sensitises them maximally This quantity is doubtless large, but is probably limited by the fact that the cell component by which the amboceptor is absorbed is itself limited The curve marked x in fig 4 also passes towards $x = \infty$ when $A = 0$, this because $\zeta = 0$ The experimental results are accordingly what we would expect, on theoretical grounds, if we were to consider the kinetics of the action of a simple lysin operating in conjunction with some agent, in this case amboceptor, which had the effect of varying the fraction of the lysin concentrated on the cells

The values of ζ and of x obtained in experiments such as these are subject, of course, to experimental errors of considerable magnitude If ζ is determined for one concentration of complement only, the experimental error may be as much as ±15 per cent It would doubtless be much smaller if several estimations could be made for several different complement concentrations and an average value obtained (as in Section 5) This, however, is impracticable in an experiment in which several different concentrations of amboceptor are used, for even the procedure described in Section 6 takes 4 or 5 hours to carry out, and the danger of the complement undergoing decay, if the experiment is further prolonged, is too great In the same way the value of x, which has to be found at the same time as that of ζ, although in a way which makes its determination really a separate experiment, is subject to an experimental error of about ±10 per cent In view of the magnitude of these possible errors, I think it is as well to regard the relations described in this paper, and especially the relation $\zeta x =$ constant, as first approximations which may subsequently require slight modification, even regarded as approximations, however, these relations provide us with a simple, consistent, and inherently probable solution of the kinetics of lysis in amboceptor-complement systems

Although **x** (*i e*, ζx) is the same for all the time-dilution curves, irrespective of the quantity of amboceptor used for sensitising the cells, k, the "velocity constant" in the equation for the fundamental reaction between cells and lysin (expression (1)), is not. As the quantity of amboceptor is increased, k becomes greater (fig 4) But it must be borne in mind that as the quantity of amboceptor increases, more and more complement is absorbed One may form a picture of what happens in the following way Some component of the cell, necessarily limited in amount, is capable of reacting with and absorbing amboceptor, the quantity of absorbed amboceptor in turn determines the quantity of complement absorbed from any given complement concentration, and it is by no means unlikely that the greater the absorption, the greater the surface of the cell involved Under such circumstances the value of k in expression (1) would be expected to become greater as the amount of absorbed complement becomes greater, both these quantities increasing with increase in the amount of amboceptor More specifically, k would be expected to be roughly proportional to ζ, whence, since $\zeta x = \mathbf{x}$ (a constant), we would expect to find the relation

$$kx = \text{constant} \tag{7}$$

This relation, however, is imperfectly fulfilled (see Table IV) The picture formed is therefore inadequate for quantitative purposes, qualitatively speaking, however, the relation is of the sort one would expect on the basis of such simple reasoning as the above

Summary

This paper deals with the kinetics of hæmolysis in systems containing anti-sheep amboceptor, sheep cells and guinea-pig complement, when the three components are added in this order

If the quantity of amboceptor is kept constant, the relation between quantity of complement used and the time required for complete hæmolysis in any given system is described by expressions identical with those for the action of a simple hæmolysin The equation describing the fundamental reaction between the complement and the cells, contains three constants, n, x, and k, the value of n, upon which the order of the reaction depends, is usually very nearly unity and the value of x and k are functions of the quantity of amboceptor used If this equation is solved simultaneously with another expression which gives the frequency distribution of the resistance of the cells to the lysin, percentage hæmolysis curves are obtained exactly as though the complement were a simple hæmolysin

It is shown that amboceptor is rapidly absorbed by the cells of the system, the quantity which is taken up being, within the experimental range at least, a constant fraction of the quantity initially introduced

In the presence of a constant amount of amboceptor, complement is also absorbed by the cells, and again the amount so absorbed is a constant fraction

of the quantity initially introduced. The taking up of complement by the sensitised cells occurs rapidly, and is complete within the first tenth of the time required for the hæmolytic reaction to be complete The numerical value of the fraction absorbed (indicated by ζ) is a function of the quantity of amboceptor used for sensitisation when the latter is great, a large fraction (*e.g.*, $\zeta = 0{\cdot}5$) of the complement is absorbed, whereas if the latter is small, the fraction absorbed is small (*e g* , $\zeta = 0{\cdot}1$)

If the quantity of complement initially introduced is C and the quantity of complement absorbed at or near the cells is C, C is thus equal to ζC Similarly, the quantity of lysin actually transformed in producing lysis of the cells is x and thus must be equal to ζx It is shown by experiment that this latter quantity is a constant, *i e*, that in the lysis of a given number of cells a constant quantity of complement is used up irrespective of the amount of amboceptor used for sensitisation The only effect which a variation in the quantity of amboceptor appears to produce is a variation in the ability of the cells to concentrate complement from the surrounding medium With a small amount of amboceptor, ζ is small and a small fraction of complement is absorbed, while with a large amount of amboceptor, ζ is large and large amounts of complement are absorbed Absorption of a small fraction from a relatively concentrated solution of complement, and absorption of a large fraction from a relatively dilute solution of complement thus result in the same thing, *i.e*, the absorption of a constant quantity and the transformation of a constant quantity x in the production of the ensuing lysis The system containing amboceptor, cells and complement can thus be treated as a system containing a simple hæmolysin, complement, which, after allowance is made for the degree to which it is absorbed at or near the cell interfaces, acts very similarly to saponin and other simple lysins

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On the True Metabolic Rate of the Chick Embryo and the Respiration of its Membranes.

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Introduction

It has always hitherto been supposed, by those who have occupied themselves with the examination of the various metabolic aspects of the developing chick embryo, that the metabolism of the embryonic membranes might be regarded as negligible Thus it has been the classical practice (as by Bohr and Hasselbalch (1900), Atwood and Weakley (1924), Lamson and Edmond (1914), Hanan (1928), and Murray (1925, *b*)) to relate the amounts of oxygen entering, or of carbon dioxide leaving, the whole egg, to the wet or dry weight of the embryo, and thus to calculate the metabolic rate, leaving the embryonic membranes out of account But it is clear that this can only be a first approximation, for the yolk-sac with its layers of endoderm and its vascular splanchnopleure must possess an active metabolism of its own The work of absorption which it performs, as illustrated, for instance, in the recent histological description of Remotti (1927), can hardly be carried out with no expenditure of energy The allantois, again, is covered with vascular splanchnopleure, and must be credited with some respiration of its own But upon precisely what this respiration amounts to relatively to that of the embryo, will depend the true values for the metabolic rate of the embryo itself It may be said, of course, that the extra-embryonic membranes consist of tissue which is physiologically just as young as that of the embryo itself, and that consequently the part played by membranes and embryo could easily be calculated from their weights, assuming their respiratory intensities to be equivalent. But to argue thus would be to make a very large assumption, an assumption which, as will later be evident from the results reported in this paper, has no foundation in fact

Until recently, measurements of the weights of the extra-embryonic membranes of the hen's egg were lacking, apart from some very incomplete data scattered in the literature (*e.g*, Hasselbalch (1900)) But the accurate work of

Byerly, as yet unpublished, has laid the foundations of a solid knowledge of the weights and growth-rates of the membranes. The weights of yolk-sac and allantois in per cent. of the total living matter in the egg are shown in fig. 1, a graph constructed from the data of Byerly. During the first 4 days of development, when the embryo is being lifted off the surface of the yolk, and the blastoderm is enclosing the yolk and the vitelline membrane, the yolk-sac forms as much as 80 per cent. of the total mass of living cells within the egg, but after this time it rapidly falls, and by the tenth day has reached a constant level which it does not afterwards forsake until the time of hatching. The yolk-sac therefore grows much more rapidly than the embryo during the first week of development, but during the last 2 weeks it grows at approximately the same rate. The allantois, on the other hand, does not begin to play an appreciable part until the fourth day, but once its growth has begun, it continues very vigorously till 9½ days of development have passed, at which time, it is outstripped by the embryo, and the allantois-weight in per cent. of the total weight, correspondingly declines, as shown in fig. 1. The curve for yolk-sac plus allantois reflects these movements.

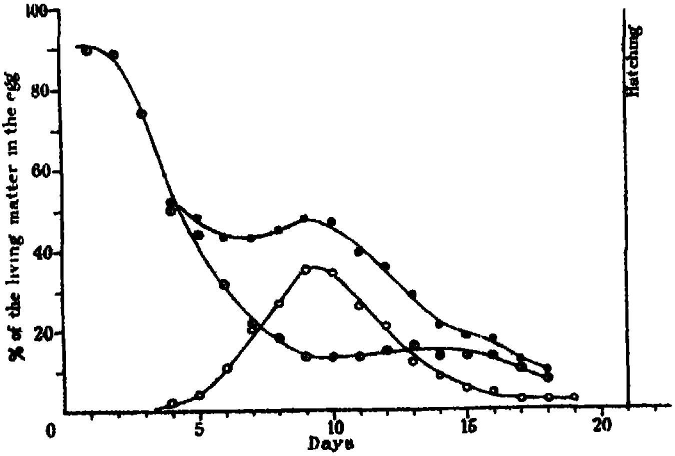

FIG. 1.

● yolk-sac,
○ allantois,
• yolk-sac and allantois together.

In estimating the significance of the extra-embryonic membranes in the metabolic life of the developing egg, the first requisite was a knowledge of their respiratory intensity. This known, a calculation of the true metabolic rate of the embryo would be possible, by subtracting the membrane respiration from the respiration of the whole egg But since the measures of efficiency of growth during embryonic life, both as regards material (the Plastic Efficiency Coefficient) and as regards energy (the Apparent Energetic Efficiency), require a knowledge of the combustion-rate of the embryo, the way would lie open for a recalculation of them in the light of more accurate information It was therefore decided to carry out a survey of the respiratory intensity of the membranes *in vitro* on each day of development

Experimental Methods

The membranes of the developing chick offer for *in vitro* respiration experiments a remarkably interesting material, which, it is surprising to notice, has never previously been studied They possess in this connection one great advantage, namely, that they may be placed in the cup of the manometer without any preliminary slicing, mincing, or destruction of cells such as is required when the ordinary masses of tissue are being studied Since they consist of thin layers of cells, it may be assumed that diffusion of oxygen is not a limiting factor in the oxygen-uptakes which they show Moreover, the membranes possess considerable structural strength, so that after 5 or 6 hours' shaking in buffered Ringer solution, the piece of yolk-sac or allantois may be removed from the cup and found to retain its original appearance This is in some contrast to the embryonic tissue itself, which shows, especially in the earliest stages, a tendency to break up into a flaky suspension

But the yolk-sac and allantois also possess one great disadvantage, namely, that in both cases it is very difficult to obtain figures for their true weight, *i.e.*, for the weight of living cells contained in them The yolk-sac, as has long been known, contains in its walls the so-called "intracellular yolk," yolk collected in small vesicles, ranging from microscopic dimensions up to well inside the range of visibility, and much more numerous in some yolk-sacs than in others Riddle (1916) is the only investigator who has taken account of the intracellular yolk, he made analyses of yolk-sacs containing it and freed from it as far as possible The allantois, again, suffers from the same disability, owing to the presence of the "amnio-allantoic cysts," first described by Boyden (1929) Boyden's cysts occur usually as transparent blister-shaped vesicles within the inner wall of the allantois in the region of the amnio-allantoic fusion. They may attain a diameter of ½ inch, but they can be found of all sizes from this downwards into microscopic regions On the amniotic side they are separated from the amniotic cavity by ectoderm, muscle, and probably mesothelium, on the opposite side they are only separated from the allantoic cavity by endoderm and perhaps mesothelium This suggested to Boyden that their relation with the allantois was probably closer than their relation with the amnion, and as they contain a viscid fluid in which coagulable protein is demonstrable, he thought it likely that they might be the source of the glairy mucous material which surrounds the precipitates of uric acid in the allantoic liquid In any case, their number and constant occurrence, together with the impossibility of removing them without destroying the integrity of the tissue, renders them the counterpart of the intracellular yolk of the yolk-sac, and makes the accurate determination of the weights of the membranes a difficult matter It is for these reasons that the scatter in the results here to be reported is much greater than might be desired Evidently only a statistical treatment could solve the point at issue

In comparing the oxygen-consumption of the isolated membranes with that of the intact egg, it was obviously very important that the membranes should be maintained in conditions as physiological as possible, and that their respiration as measured *in vitro* should reproduce as accurately as possible their respiration in the intact egg It would therefore have been desirable to use the Warburg manometer, since the bicarbonate Ringer solution and gas mixture employed in it may probably be considered more physiological than the phosphate buffer and Ringer solution of the Barcroft differential manometer But certain experiments (Nos 116, 117 and 118, not included in the subsequent tables) showed that the Warburg apparatus was not suitable The membranes possess, for their weight, a low respiratory intensity, and quantities of less than 50 mgm —the maximum weight of tissue permissible in the Warburg cup—gave oxygen-uptakes too small in comparison with the temperature changes to allow of trustworthy readings being taken The Warburg apparatus is, of course, uncompensated However, it has recently been shown by Dickens and Simer (1931 *b*) that the respiratory quotients and respiratory intensities of a large variety of tissues (including the chick embryo) are exactly the same whether determined in phosphate or bicarbonate, and this provides a sound justification for the present experiments, carried out as they were in Barcroft manometers

Incubation of the hen's eggs was done according to the standard conditions laid down by Murray (1925, *a*) and Needham (1926), that is to say, temperature 38·8 ± 0·5° C., humidity 67·5 ± 2 5 per cent, and continuous ventilation They were all derived from the pure-bred flock of White Leghorn hens owned by Messrs Chivers, of Histon, Cambridgeshire, a fact of some importance, as the work of Murray (1925, *b*) on the oxygen-consumption of the intact egg was carried out on eggs from this same breed Moreover, this same flock had provided eggs during the previous 10 years for the extended work of Needham (1926, 1927, 1928) and Murray (1925, *a* and *b*) on the metabolism of the proteins, fats, and carbohydrates in the developing egg

The removal of the yolk-sac and allantois presented no special difficulty except the separation of adhering liquids—yolk and allantois contents It was found that the allantois could be easily freed from allantoic liquid by gentle squeezing and cautious treatment with filter-paper, but the yolk-sac required a different technique, especially during the later stages when the vitelline blood-vessels are slung away from the yolk-sac wall on little mesenteries, the spaces between which are full of yolk The procedure which answered best was to drag the yolk-sac, internal face down, over a large expanse of dry clean plate glass A certain amount of fragmentation during this was unavoidable, but in any case, division of the yolk-sac had to take place quite soon in development, one yolk-sac being too large for the manometer The cleansing of the membranes by washing in saline would, of course, have been easy, but the danger of washing away metabolites precluded this A few experiments were made with washed membranes (see Table I, experiments 3, 10 and 109, and Table II, experiment 75), and a few others with washed membranes to which yolk was added before the experiment (see Table I, experiments 94 and 110) Comparing these with their immediate neighbours, a decreased respiration can be observed in the case of the washed membranes in most cases Addition of yolk appeared to be effective in abolishing this decrease, but with the allantois, one would be at a loss to know what material to add, except the embryonic blood The question merited further study, but in view of the labour involved in the main part of the investigation, it was thought better to proceed by avoiding washing

The problem of p_H was difficult, and indeed logically insoluble, since the membranes in the intact egg are subjected to media of very varying hydrogen ion concentration On the seventeenth day, for example, the allantois has the allantoic liquid, of p_H 6 0, on one side of it, the amniotic liquid, of p_H 5·0, on the other side of it, and blood, of p_H 7 2, passing through its vessels Or again, the yolk-sac on the fifth day has yolk of p_H 5 8 within it, white of p_H 8·3 outside it, and blood of about p_H 6·8 in its vascular system (authorities may be found in Needham (1931)). In these circumstances it was impossible to select a characteristic p_H for each membrane, and two buffer solutions were made up, at p_H 5·8 and 7·0, estimations being in many cases duplicated. It is evident from Tables I, II and III and figs 6 and 7 that no significant statistical difference was found between the respiratory rates at these two p_H The experiments in the Warburg manometer were of necessity done at p_H 7·6—another disadvantage of the Warburg technique for this particular problem, since the liquids of the egg tend on the whole to be on the acid side of neutrality, with the great exception of the egg-white, and even the egg-white has reached neutrality by the thirteenth day of development

The Barcroft manometer technique followed closely the lines laid down by Dixon and Elliott (1930) in their standard discussion of this instrument, as regards carbon dioxide absorption, rate of shaking, etc The relation between the observed uptake of oxygen and the weight of tissue present gives a test of the validity of the method, and should, of course, be linear, since the velocity of uptake of oxygen should be proportional to the weight of respiring protoplasm If the oxygen uptake shows any tendency to become independent of the weight of tissue, diffusion has become a limiting factor Fig 2, constructed

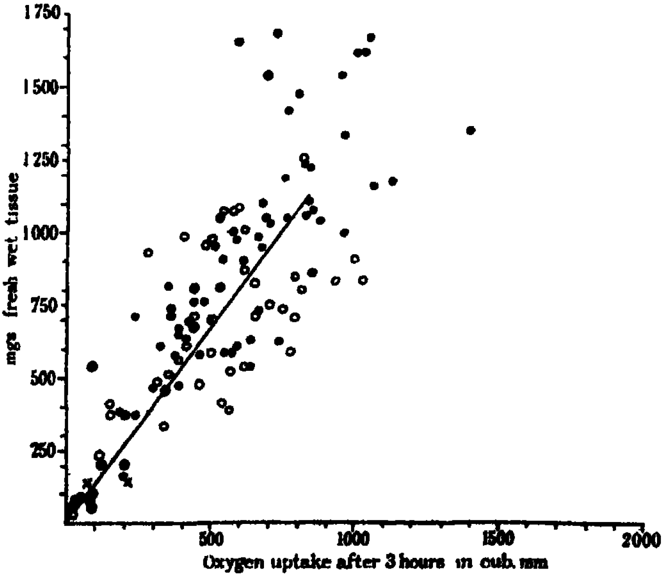

FIG 2

- • yolk-sac ,
- o allantois ,
- x amnion ,
- ● blastoderm ,
- ● embryo

from the whole of the data here collected, shows that the linear relation holds well up to 1200 mgm of membrane, but it is probable that above that point the results are not reliable Another valuable test was afforded by the fact that experiments 112, 113, 114 and 115 (Table II), which were done in atmospheres of 95 per cent oxygen instead of air, gave results which fell well within the usual range for membranes of the age taken Some typical uptake curves are shown in fig 3

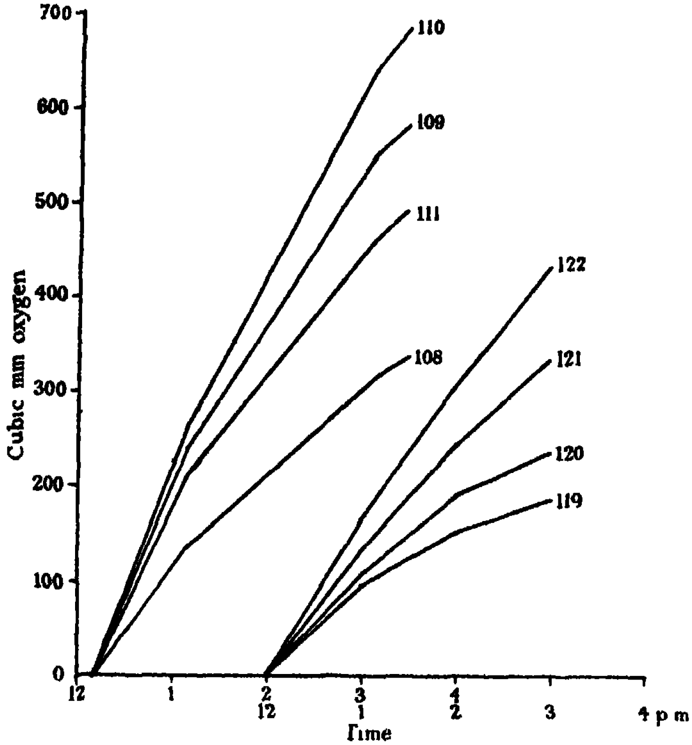

Fig 3 —Oxygen uptakes of yolk-sacs and allantoic membranes *in vitro*.

In order to be able to apply Byerly's data for the percentage participation of membranes in the total live weight of the egg (see fig. 1) to the present data with confidence, a comparison was made between the two sets of figures for actual weights of yolk-sac and allantois In fig 4 are shown the weights of yolk-sacs here reported and the zone of weight established by Byerly—the correspondence is obviously fairly close, although there is shown throughout a tendency to be smaller or lighter than the American series. The same result is found when the two sets of figures for the allantois are plotted, as in fig. 5, but here the difference is much more marked, and amounts indeed to between

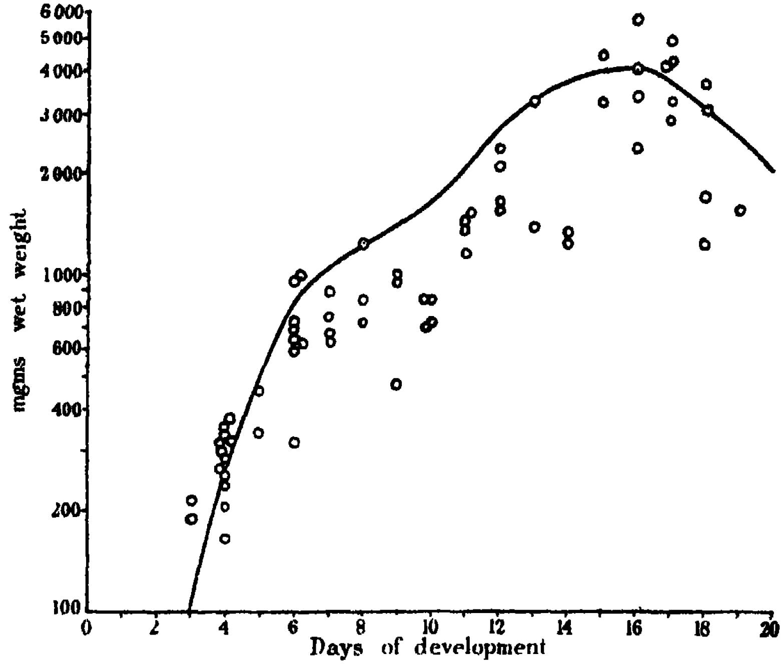

FIG 4—Growth of yolk-sac, comparison of data here reported with those of Byerly The semilog scale permits of all the figures being plotted together

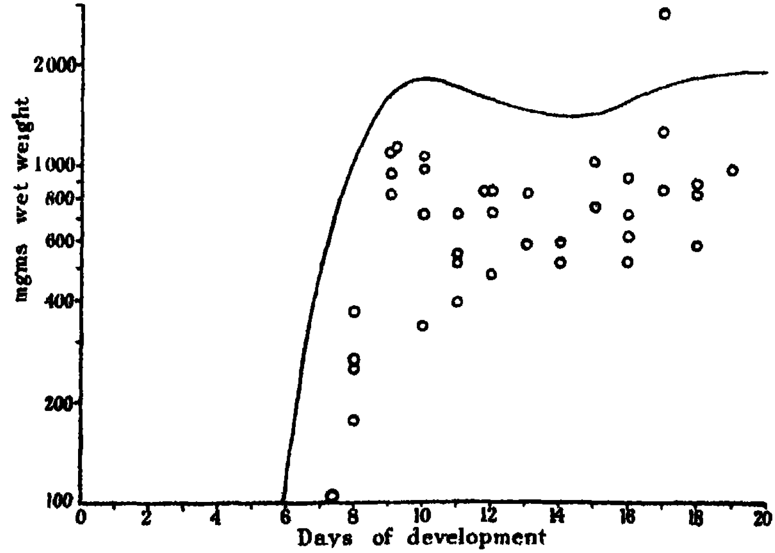

FIG 5—Growth of allantois comparison of data here reported with those of Byerly. The semilog scale permits of all the figures being plotted together

40 per cent and 50 per cent This must certainly be regarded as a difference of breed, either leading directly in the case of the Cambridge flock of White Leghorns to a thinner allantoic wall, or by way of some intermediate factor such as the frequency and size of Boyden's cysts, diminishing its weight These differences will be taken into consideration later in discussing the calculations of the true metabolic rate of the embryo

Experimental Results

The results for the respiratory intensity of isolated yolk-sacs are summarised in Table I, where the day of development, p_H, and oxygen-consumption in cubic millimetres per hour per gram is given Table II gives the same summary in the case of the allantois, and Table III adds a few experiments with amnion, whole blastoderm (including the embryonic area), and the later embryo itself The amnion, as appears from experiments 50 and 105, has about the same respiratory rate as the allantois, but in view of the very small ponderal part which it plays in the egg, it has here been neglected for the sake of simplicity As regards the embryo, the figures are rather scattered, but seem to indicate a fall from 700 c mm /gm /hr on the third day to 150 c mm /gm /hr on the seventh As will be shown later, however, these figures fall very much below the calculated level of metabolic rate for the embryo, which would be about 1200 c mm /gm /hr on the sixth day, and no great importance can be attached to them in view of the difficulty of diffusion of oxygen into the interior of a lump of tissue such as a seventh day chick embryo It is, however, possible that the figures for third and fourth day embryos are substantially correct, this possibility will be referred to again

Table I —Respiratory Intensity of Isolated Yolk-sacs

Experiment No	p_H	Day of development	Weight used (mgm)	Oxygen consumption cub mm /hr for first 2 hours	Weight of whole yolk-sac. (mgm.)
2	5 8	4	164	391	164
3	5 8	4	210	183	210
6	5 8	6	630	358	630
7*a*	5 8	6	599	334	599
7*b*	—	6	703	—	703
7*c*	–	6	641	—	641
7*d*	—	6	688	—	688
8	5 8	6	626	394	313
9	5 8	7	89	364	890
10	5 8	2	36	139	9
11	5 8	7	62	313	620
13	5 8	8	608	388	1216
14	5 8	3	557	270	188

Table I.—(continued).

Experiment No	p_H	Day of development	Weight used (mgm)	Oxygen consumption cub mm /hr for first 2 hours	Weight of whole yolk-sac (mgm)
16	5 8	3	650	257	216
17	5 8	9	473	317	473
29	5 8	4	749	234	249
30	5 8	4	1107	208	369
31	5 8	4	633	394	316
34	5 8	6	999	360	999
35	5 8	6	962	230	962
36	5 8	5	900	241	450
37	5 8	5	680	251	340
40	5 8	4	984	275	328
41	5 8	4	1033	271	344
43	5 8	4	1056	294	352
44	5 8	7	1005	240	671
45	5 8	7	1119	305	746
48	5 8	8	1234	264	844
49	5 8	8	1078	283	719
52	5 8	9	978	261	978
53	5 8	9	950	295	950
56	5 8	18	1672	269	1672
57	5 8	18	1221	270	1221
60	5 8	19	1541	211	1541
64	5 8	10	1059	274	706
65	5 8	10	1064	349	710
68	5 8	16	1695	177	3390
69	5 8	16	1191	268	2382
72	5 8	17	1428	281	4280
73	5 8	17	1657	159	4970
77	7 0	11	1167	364	1167
78	7 0	11	1350	432	1350
81	7 0	12	1040	404	2080
82	7 0	12	1177	357	2354
85	5 8	13	1625	301	3250
86	5 8	13	692	289	1384
89	7 0	14	655	290	1310
90	7 0	14	615	244	1230
93	7 0	15	1489	262	4460
94	7 0	15	1630	272	3260
97	7 0	16	1343	313	4040
98	7 0	16	1914	257	5720
101	7 0	17	1058	208	4220
102	7 0	17	822	201	3290
103	7 0	17	710	141	2840
106	7 0	18	904	232	3620
107	7 0	18	769	254	3060
108	7 0	4	479	262	239
109	7 0	4	584	369	292
110	7 0	4	537	447	268
111	7 0	4	570	333	285
119	7 0	10	374	233	748
120	7 0	10	374	273	748
124	5 8	11	734	238	1468
126	7 0	11	723	400	1446
127	5 8	12	769	261	1538
129	7 0	12	866	404	1632

N B.—Experiments 3, 10 and 109, membranes washed in saline, experiments 94 and 110, membranes washed and 1 c.c of yolk added before the experiment

Table II —Respiratory Intensity of Isolated Allantoic Membranes

Experiment No	p_H	Day of development	Weight used (mgm)	Oxygen consumption (cub mm /hr for first 2 hours)	Weight of whole allantois (mgm)
18	5 8	9	561	267	1122
20	5 8	9	406	191	812
32	5 8	6	237	160	59
39	5 8	5	30	–	4
47	5 8	7	270	241	90
51	5 8	8	1084	176	362
54	5 8	9	945	117	945
55	5 8	9	1082	250	1082
58	5 8	18	878	285	878
59	5 8	18	585	324	585
61	5 8	19	972	196	972
66	5 8	10	985	158	985
67	5 8	10	1077	184	1077
70	5 8	16	520	395	520
71	5 8	16	613	253	613
74	5 8	17	1257	286	1257
75	5 8	17	964	199	2890
76	7 0	11	545	405	545
79	7 0	11	708	367	708
80	7 0	12	835	443	835
83	7 0	12	833	396	833
84	5 8	13	823	316	823
87	5 8	13	482	239	482
88	7 0	14	408	503	408
91	7 0	14	593	472	593
92	7 0	15	756	371	756
95	7 0	15	1005	298	1005
96	7 0	16	710	324	710
99	7 0	16	915	383	915
100	7 0	17	848	311	848
104	7 0	18	803	396	803
112	7 6	8	41	195	246
114	7 6	8	44	308	265
115	7 6	8	29	103	174
121	7 0	10	332	382	332
122	7 0	10	713	224	713
123	5 8	11	516	272	516
125	7 0	11	399	500	399
128	5 8	12	471	382	471
130	7 0	12	735	360	735

N B.—Experiments 114 and 115 were done with unbuffered Ringer solution at p_H 7 6, experiment 112 was done in phosphate buffer at p_H 7 6 without Ringer, experiment 75 was done on a membrane previously washed in saline

There have been several previous attempts to measure the respiration of chick embryos directly *in vitro* The first was that of Shearer (1924) who separated them between the fourth and tenth day of incubation into two (presumably equal) portions, head and tail, and measured their oxygen-uptake He expressed his results in terms of the amount of tissue containing 1·4 mgm. nitrogen, but by means of the subsequent work of Murray (1925)

Table III —Respiratory Intensity of Isolated Amnion, Whole Blastoderm and Embryo

Experiment No	p_H	Day of development.	Weight used (mgm)	Oxygen consumption (cub mm./hr for first 2 hours)	Weight of whole (mgm)
		Amnion			
50	5 8	8	138	202	46
105	7 0	18	141	525	141
		Whole Blastoderm			
25	5 8	2 75	530	114	132
27	5 8	2 75	810	222	270
		Embryo			
1	5 8	4	207	442	29
5	5 8	6	810	219	202
15	5 8	3	51	649	5
24	5 8	2 75	80	162	20
26	5 8	2 75	89	214	22
28	5 8	4	370	135	46
33	5 8	6	1239	113	413
38	5 8	5	700	242	80
42	5 8	4	455	297	45
46	5 8	7	1545	168	515

and Needham (1927, *b*) this can be translated into terms of embryo weight When this is done it is seen that something must have been seriously amiss with Shearer's experiments, in that on the sixth day his embryos only took up 0·7 per cent of the oxygen which they should have taken up, judging from the oxygen-intake of the whole egg corrected for the membrane-respiration, on the seventh day only 0 3 per cent , on the ninth day and the tenth day only 0 2 per cent Indeed, in a later paper Shearer withdrew his previous conclusions (1930), "fresh experiments have since convinced me," he wrote, "that I was dealing with very abnormal conditions of respiration in these experiments " Thus the respiration ceased abruptly after 30 minutes and the disintegration of the embryos was considerable But a full explanation is still lacking, for Shearer finally regarded his results as being artificially raised by cytolytic processes, whereas when they are compared with the respiration of the intact egg, they are seen to be infinitesimally small

Results much more in harmony with those here reported are to be found in the papers of Kayser, Le Breton and Schaeffer (1925), Warburg, Posener and Negelein (1924), Kumanomido (1928), and Dickens and Simer (1930, 1931) These may be summarised as follows —

Day of development	Author and conditions	Q_{O_2} (cub mm / hr /mgm dry weight)	Embryo dry weight (mgm)	Oxygen consumption (cub mm /hr /gm wet weight)
5	Kayser, Le Breton and Schaeffer (1925) Ringer		18	740
4	Warburg, Posener and Negelein (1924) Bicarbonate Ringer 0 2 per cent glucose, p_H 7 6	10 0	2 7	338
3 5	Kumanomido (1928) Hen serum	9 3	4 3	398
4	Dickens and Simer (1930, a) Phosphate Ringer, 0 2 per cent glucose, p_H 7 4	9 5	11 5	414
		7 9	14	419
4	Dickens and Simer (1931, b) Bicarbonate Ringer, 0 2 per cent glucose, p_H 7 6	11 3	4 1	351
		11 6	3 1	272

These figures are of about the same order as those reported here in Table III

The data for yolk-sac and allantois are plotted in figs 6 and 7 The former maintains a steady level of respiratory rate from the third day, when the readings

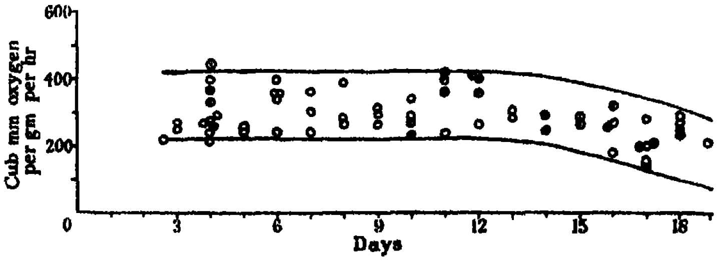

Fig 6 —Respiratory rate of yolk sac

○ p_H 5 8 in air,
● p_H 7 0 in air

begin, until the thirteenth day, after which point it falls off and has suffered a diminution of about 40 per cent by the time of hatching If this be compared with the curve of fig 4, it will be observed that, during the whole period of its active growth, the respiratory rate of the yolk-sac is steady, but its maximum weight is reached on the sixteenth day and is followed by a decline, corresponding with the decline in its respiratory rate These relations offer an interesting picture of the coming-into-being and passing-away of the primary piece of scaffolding in the meroblastic egg A similar close relationship is noticeable between the respiratory rate of the allantois and its growth, for during the period of its rapid development (the fifth to the eleventh day, see figs 7 and 5) its respiratory rate is rising too, but after this time the weight of the allantois remains practically steady and its respiratory rate slowly falls off

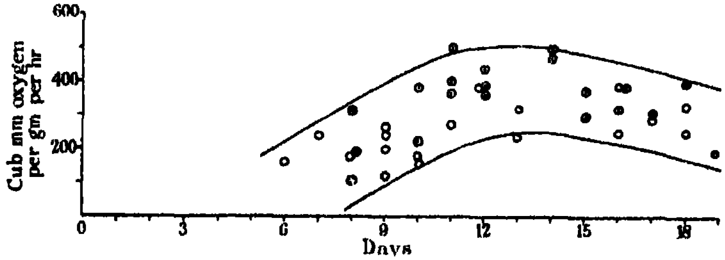

Fig 7 —Respiratory rate of allantois

○ p_H 5·8 in air,
◉ p_H 7·0 in air,
● p_H 7·6 in oxygen

Discussion

(*a*) *The True Metabolic Rate of the Embryo* —The justification for comparing the figures for membrane respiration here reported with those for the respiration of the intact egg obtained by Murray (1925, *b*), instead of making a new series of such observations, was that Murray's work was largely done, not only with the same breed, but with eggs from the same flock of hens The use of other sets of data, such as those of Atwood and Weakley (1924), would certainly be more hazardous Calculations of the embryonic metabolic rate are shown in Tables IV and V As a by-product of these calculations, the percentage participation of the membranes in the respiration of the whole egg was obtained —it is plotted in fig. 8. By comparison with fig 1 an exact assessment of the relative importance of the extra-embryonic membranes can be had Fig 9 gives the oxygen-consumption of the yolk-sac and allantois plotted against time, and here again the relation between respiratory rate and growth-rate

Table IV.—Calculation of the True Metabolic Rate of the Chick Embryo

Day of development	Oxygen-consumption in cub. mm./gm./hr		Wet weights, mgm (Byerly)			Oxygen-consumption in cub. mm./hr		
	Yolk-sac (see fig. 6).	Allantois (see fig. 7)	Yolk-sac	Allantois	Embryo	Yolk sac (see fig. 9)	Allantois (see fig. 9)	Yolk-sac and allantois together
1	—	-	—	—	—	—	—	—
2	—	—	—	-	-	—	-	—
3	320	-	102	—	29	33	-	33
4	320		265	4	132	88	—	88
5	320	—	477	23	264	153	—	153
6	320	63	870	120	562	278	7	285
7	320	130	1029	508	901	330	66	396
8	320	184	1244	1052	1488	399	194	593
9	320	240	1368	1595	2068	437	383	820
10	320	294	1790	1838	3168	572	529	1101
11	320	342	2098	1692	4304	670	580	1250
12	320	373	2877	1580	6100	920	590	1510
13	316	381	3170	1410	8555	1000	538	1538
14	310	377	3688	1527	11838	1140	577	1717
15	295	360	3823	1330	14320	1128	480	1608
16	272	342	4014	1514	17570	1093	518	1611
17	238	320	4100	1776	21870	980	567	1547
18	220	295	2643	1745	24210	580	515	1095
19	180	270	2813	1826	28270	507	494	1001
20	—	—	1840	—	35690	—	—	—

Table V—Calculation of the True Metabolic Rate of the Chick Embryo—(continued)

Day of development	Oxygen consumption of whole egg in cub mm./hr (Murray (1925, *b*))	Oxygen consumption of yolk-sac and allantois in per cent of the oxygen-consumption of the whole egg (per cent)	Oxygen consumption of embryo alone (cub mm /hr)	Metabolic rate (Oxygen-consumption of embryo in cub mm /hr /gm)		
				Corrected for membranes		Not corrected.
				Weight data of Murray (1925, *a*)	Weight data of Byerly (unpub)	
1				—	—	—
2				—		—
3	—	—		—	—	—
4		—	—	—	—	—
5		—			—	—
6	880	32 3	595	1400	1057	2080
7	1265	31 4	869	1190	962	1790
8	1950	30 5	1357	1130	913	1620
9	2910	28 2	2090	1090	1015	1520
10	3820	28 8	2719	1040	858	1460
11	5300	23 6	4050	1085	940	1415
12	6970	21 7	5460	1090	893	1380
13	9800	15 7	8262	1140	967	1355
14	12500	13 5	10783	1140	906	1320
15	14900	10 8	13293	1135	930	1270
16	17200	9 4	15600	1090	889	1200
17	20000	7 8	18450	1008	846	1090
18	20200	5 4	19100	917	790	967
19	21900	4 6	20900	790	740	835
20	—	—	—	—	—	—

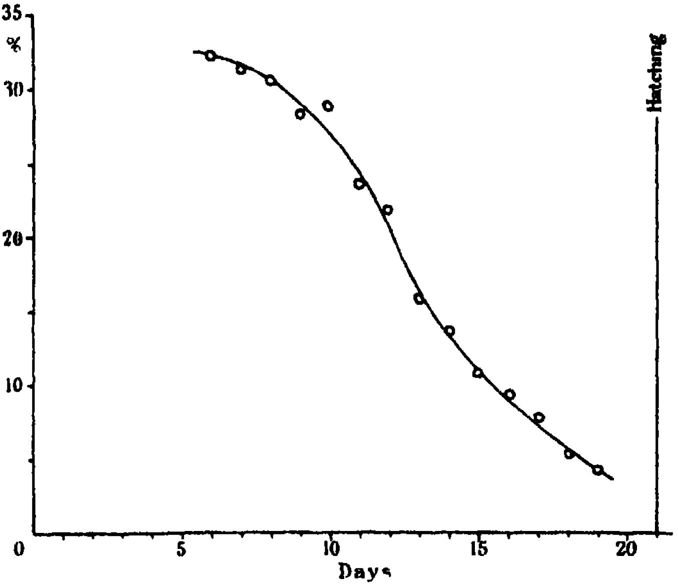

Fig 8.—Respiration of yolk-sac and allantois in percentage of the respiration of the whole egg

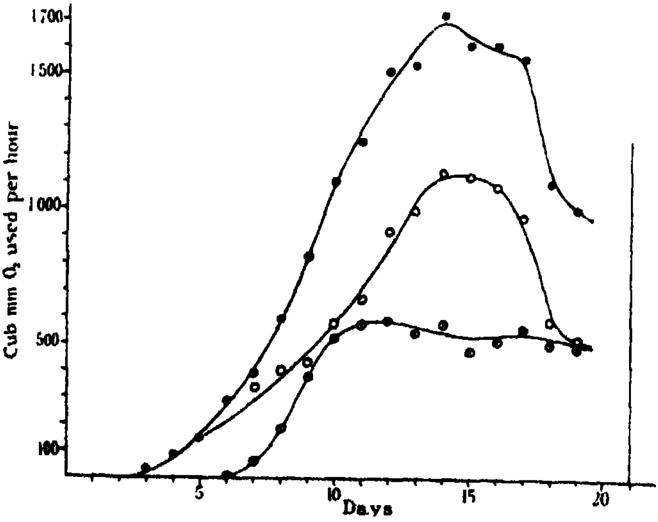

Fig 9

● yolk-sac and allantois together,
○ yolk-sac;
◉ allantois

can be found by comparing fig 9 with figs 4 and 5 The total respiration of the extra-embryonic membranes shows a striking peak on the fourteenth day of development. Finally, fig. 10 shows the apparent metabolic rate of the embryo, and the same when corrected for the respiration of the membranes.

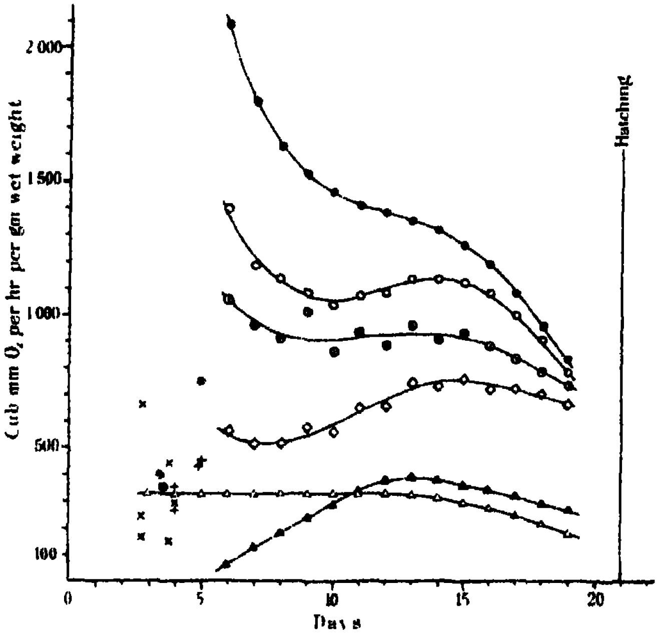

FIG 10 —Metabolic rate

Embryo

● Curve uncorrected for respiration of membranes (Murray)

○ Curve corrected for respiration of membranes (Murray's embryo weights)

● Curve corrected for respiration of membranes (Byerly's embryo weights)

× Observed values obtained manometrically (Needham)

● ,, ,, ,, (Kayser)

● ,, ,, , (Warburg)

● ,, , , (Kumanomido)

+ ,, ,, , (Dickens and Simer)

Yolk sac

△ Curve of manometric observations (Needham)

Allantois

▲ Curve of manometric observations (Needham)

Embryo, Yolk-sac, and Allantois together

○ Curve calculated from oxygen-consumption of intact egg (Murray), growth of the membranes (Byerly), and respiration of the membranes (Needham)

As was to be expected, since the younger embryos had been credited with a great deal of respiration that was not theirs, the earlier parts of the curve are depressed, and the later parts very little affected Some difference appears according to whether the embryo weights of Murray or Byerly are used in the calculation, but this is not very considerable And a fall in the embryo's metabolic rate is still seen, although reduced from 57 2 per cent to 33 4 per cent

The peculiar importance of an accurate evaluation of the chick's respiratory intensity lies in the fact that we cannot as yet make any generalisation about metabolic rate in embryonic life, although there are indications which show what form this generalisation should take Firstly, for echinoderm development, all workers agree that the respiratory rate steadily rises from fertilisation onwards (Warburg (1910), Shearer (1922), Gray (1925), Rapkine (1927)) and that the rate of heat-production also rises (Meyerhof (1911)) Molluscan embryos, according to Buglia (1908), also show a rising metabolic rate, and the same process has been abundantly demonstrated for amphibian embryos by Parnas and Krasinska (1921) and Godlevski (1901)

On the other hand, in mammals, it has been classical to speak of the fall of metabolic rate with age, especially in post-natal stages (Magnus-Levy and Falk (1899), Krarup (1902), and others) And the apparent metabolic rate of the chick embryo, with its considerable fall, as given by Bohr and Hasselbalch (1900) and by Murray (1925, *b*), fitted into this scheme It could therefore have been said that in the cases of those animals where the late stages of embryonic life had been investigated, the metabolic rate was observed to fall with age, while in those cases where the early stages of embryonic life had been investigated, the metabolic rate was observed to rise. There was therefore strong presumptive evidence for the existence of a peak of metabolic rate somewhere in the obscure regions of embryonic life And the belief in the existence of such a peak or maximum was considerably strengthened by the reflection that if the descending metabolic rate curve of a mammal were merely extrapolated backwards in time, the embryo of a few grams weight would be radiating heat with the intensity of a red-hot body

The first work which showed clearly the existence of the predicted peak was that of Gayda (1921), who studied the heat-production of toad embryos and larvæ for some 4 months after fertilisation The heat-production in calories per gram per hour rose steadily without an inflection at hatching to reach a maximum at the twentieth day This suggested that the course of metabolic rate in the human infant might be similarly interpreted Dubois (1916), in his classical paper on the change of metabolic rate with age, noted a peak at 5 years in man, and pointed out that if this peak were real, infants born some time before term would be expected to show a basal metabolism lower than that of normal infants This was actually found to be the case by Murlin and Marsh (1922), Marsh (1923), Talbot and Sisson (1922), and Talbot, Sisson, Moriarty, and Dalyrymple (1922, 1923) Again, Wood (1926) found a very similar peak of basal metabolism in the case of the pig, depending upon breed for its exact situation, whether just before or just after birth And Ginglinger and Kayser (1929) have observed a post-natal peak in the rabbit, with evidences of a pre-natal one in the guinea-pig

These facts lead to the conclusion that a maximum of metabolic rate must exist at some point during the development of the chick embryo, but that this point must occur before the sixth day As has already been stated, a few figures for the respiratory rate of young embryos alone *in vitro* are available and these are accordingly introduced into fig 10 There is evidently agreement between those of the present paper and those obtained in the course of other investigations by Kayser, Le Breton and Schaeffer (1925), Warburg, Posener and Negelein (1924), Kumanomido (1928), and Dickens and Simer (1930 and 1931), but it is difficult to calculate from their descriptions, since often the wet weight of the embryos and their age are not given If we accept these estimations as reliable, we must assume that the peak of metabolic rate occurs actually on the fifth day, but a special investigation will be required to establish its exact position In particular we lack accurate measurements of the oxygen consumption of the intact egg before the sixth day, no doubt because the technical difficulties are here so acute, where very small changes in gas distribution occur in association with a very large mass of inert material

In order to facilitate the comparison of metabolic rate in embryo and extra-embryonic structures, the data here collected for allantois and yolk-sac are also inserted in fig 10 The differences between the weights of membranes in this work and those in Byerly's may here be recalled It is curious that the membrane which showed most difference is also the one which is ponderally the less important, namely, the allantois, so that if in fig 1 the allantois figures were to be reduced by anything up to 40 per cent, the curve for the total membranes would not be very greatly affected To this modification, however, we have to add a diminution of some 15 per cent in the yolk-sac curve (see figs. 1 and 4) bearing particularly upon the period between the seventh and thirteenth days The embryonic metabolic rate curve in fig 10 is therefore probably slightly over-corrected throughout, but especially in the region between the seventh and thirteenth days, *i.e.*, in exactly the region where a trough appears in it It is probable, therefore, that this trough has no significance, and that the curve for true metabolic rate should descend from 1200 to 800 c mm /gm /hr in a regular sigmoid curve, involving no rise even of a temporary nature

In addition to the metabolic rate of the embryo and the metabolic rate of the membranes, it is possible to calculate that of the whole mass of living material within the egg, although this is not of such great interest, as it will simply reflect the rates of the constituents according to their ponderal dominance. The curve for the overall metabolic rate in fig 10 is first of all concave upwards and then concave downwards It does little more than demonstrate that the membranes, with their low rate of respiration, are dominant at the beginning of development, while the embryo, with its high rate of respiration, is dominant at the end

(*b*) *The Plastic Efficiency Coefficient*.—The degree of efficiency with which the *transference of yolk and albumen into flesh and blood is effected may most* conveniently be expressed by an efficiency coefficient The ratio of wastage to storage, when applied to the material constituents of the egg, is easily calculated by dividing the dry weight of the embryo by the dry weight of absorbed solid at any given moment It is usually called the Plastic Efficiency Coefficient, and was calculated for the chick embryo in 1927 by Needham (1928) A recalculation in view of the data here reported was evidently desirable, since the respiration of the membranes was previously wrongly attributed to the embryo The necessary data are given in Table VI and the result in fig 11 Evidently the efficiency of the embryo alone is greater throughout development than had previously been supposed, but the general shape of the curve remains unchanged, and shows the depression at 8·5 days of development which coincides with the point of maximum protein catabolism and maximum excretion of waste nitrogen.

But the efficiency of the membranes alone can now also be calculated, since their respiration is known This is accomplished in Table VIII, and the results are plotted in fig 11 As the membranes stop growing about the fourteenth day, their storage after that point is nil, but their respiration continues, and their efficiency of growth falls therefore to nothing. This appears well in fig. 11, but what is more remarkable is their extraordinarily high efficiency during the period of their rapid growth, *i e*, before the twelfth day, here no less than 90 per cent of the absorbed material, even up to 97 per cent., is

Table VI —Plastic Efficiency Coefficient (Embryo)

Interdiurnal period.	Increments of dry weight.				P E.C. (embryo alone)
	Storage (data of Murray (1925,*a*) and Needham (1927, *c*)) (mgm)	Combustion (data of Murray (1925,*b*) from oxygen intake of intact egg) (mgm),	Combustion corrected for percentage participation of membranes (Table V, column 3) (mgm)	Absorption (col 2+col. 4) (mgm)	
1– 2	—	-		—	—
2– 3	-		—	—	—
3– 4	3	1	—	—	—
4– 5	7	3	-	—	—
5– 6	12	6	—	—	—
6– 7	19	11	7·5	26·5	0·73
7– 8	31	20	13·8	44·8	0·69
8– 9	44	32	22·6	66·6	0·66
9–10	68	45	32·2	100·2	0·68
10–11	102	60	34·3	136·3	0·75
11–12	161	80	61·9	222·9	0·73
12–13	241	105	85·4	326·4	0·74
13–14	409	132	113	522	0·78
14–15	575	164	144	719	0·80
15–16	686	198	178	864	0·79
16–17	730	236	216	946	0·77
17–18	797	253	236	1033	0·77
18–19	832	259	246	1078	0·77
19–20	—	-	—	—	—

transformed into living cells This is demonstrated even more strikingly by the efficiency curve for embryo and membranes together, calculated in Table VII and plotted in fig 11 It begins earlier than the curve for membranes alone because Murray's data for oxygen-intake of the whole egg begin 2 days earlier than the data here reported for oxygen-consumption of the isolated membranes This curve, the Plastic Efficiency Coefficient each day for the whole living matter in the egg, is at first extremely high, owing to the great efficiency of the membranes, and the fact that at that time they are ponderally much the most important part of the living system But it falls as their efficiency falls, and at the end of development occupies a position close to that of the efficiency of the embryo alone, for the embryo now has replaced the membranes as the largest part of the system The reason why it dips below the curve for the embryo alone is due simply to the fact that Byerly's figures for weight of embryo (Table VII, column 3) were used in the calculation instead of Murray's (Table VII, column 2), and the former show much more markedly the well-known cessation of growth towards the time of hatching than do the latter

What explanation may be advanced for the remarkably high efficiency of the membranes during the early stages is not clear, and the subject merits further examination, of which the cytological study of the cells of the yolk-sac would form a valuable part The maintenance metabolism of the cells of the yolk-sac during the first week of development must be extremely small, and it is therefore relatively far cheaper to build what is then built, than to construct the complicated systems of later development It is important not to confuse here the efficiency calculated by comparing combustion and storage with that which could be calculated if the basal metabolism which each cell

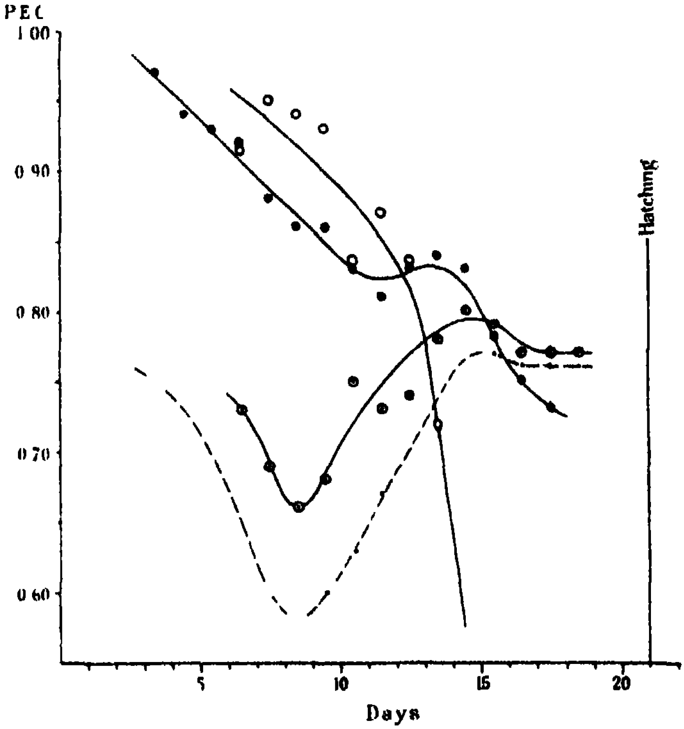

Fig 11 —Plastic Efficiency Coefficients

----- old values for embryo ,

⊙ new values for embryo ,

○ membranes alone ,

• embryo and membranes together

Table VII.—Plastic Efficiency Coefficient (Embryo plus Membranes)

Interdiurnal period.	Increments of dry weight (mgm.)								P.E.C. (embryo and all membranes).
	Storage						Combustion	Absorption.	
	Embryo (Murray (1925, *a*)).	Embryo (Byerly (unpub)).	Yolk-sac (Byerly (unpub))	Allantois (Byerly (unpub)).	Yolk-sac and allantois together	Embryo and all membranes (Byerly (unpub))	Whole egg (Murray (1925, *b*)	(Col. 7 + col 8))	
1– 2	—	—	—	—	—	—	—	—	—
2– 3	—	1 4	16	0	16	17	—	—	—
3– 4	3	6	29	0	29	35	1	36	0 97
4– 5	7	9	44	1	45	54	3	57	0 94
5– 6	12	21	60	5	65	86	6	92	0 93
6– 7	19	27	80	17	97	124	11	135	0 92
7– 8	31	33	100	26	126	159	20	179	0 88
8– 9	44	42	110	43	153	195	32	227	0 86
9–10	68	68	130	42	172	240	45	285	0 86
10–11	102	168	100	24	124	292	60	352	0 83
11–12	161	212	110	12	122	334	80	414	0 81
12–13	241	405	90	10	100	505	105	610	0 83
13–14	409	647	50	0	50	697	132	829	0 84
14–15	575	820	0	0	0	820	164	984	0 83
15–16	686	710	0	0	0	710	198	908	0 78
16–17	730	700	0	0	0	700	236	936	0 75
17–18	797	690	0	0	0	690	253	943	0 73
18–19	832	330	0	0	0	330	259	—	—
19–20	—	—	0	0	0	—	—	—	—

N B.—The figures in columns 3, 4 and 5 are all derived from curves smoothed through Byerly's data.

Table VIII.—Plastic Efficiency Coefficient (Membranes alone)

Interdiurnal period	Increments of dry weight (mgm.)			P E C (membranes alone)
	Storage all membranes (from Table VII, col 6)	Combustion due to membranes (calculated from Table V, cols. 2 and 3)	Absorption (col 2 + col 3)	
1– 2		—	—	—
2– 3	16	—	—	—
3– 4	29	—	—	—
4– 5	45	—	—	—
5– 6	65	—	—	—
6– 7	97	3 5	100 5	0 92
7– 8	126	6 2	132 2	0 95
8– 9	153	10	163	0 94
9–10	172	13	185	0 93
10–11	124	26	150	0 83
11–12	122	18	140	0 87
12–13	100	20	120	0 83
13–14	50	19	69	0 72
14–15	0	20	20	0 00
15–16	0	20	20	0 00
16–17	0	21	21	0 00
17–18	0	17	17	0 00
18–19	0	11	11	0 00
19–20	0	—	—	—

must undertake as soon as it is formed, were accurately known. A fuller discussion of these problems is given elsewhere (1931, Section 7)

(c) *The Apparent Energetic Efficiency*.—In just the same manner as an estimate can be had of the efficiency in material growth, so also growth in terms of energy has its efficiency By dividing the chemical energy stored by the chemical energy absorbed, the apparent energetic efficiency is obtained The operation of correcting the previous values for the activity of the membranes is somewhat hazardous, for as yet we know nothing of the material combusted by the membranes and we cannot therefore say what its calorific value is at any moment It is further impossible to calculate the Apparent Energetic Efficiency for the membranes alone, for we know nothing of their calorific value However, a tentative correction of the data for the embryo alone is given in Table IX and fig 12. As was to be expected, the values are raised, especially in the early stages It will be interesting to see whether in further work the values for Apparent Energetic Efficiency before the sixth day turn out to be higher than those at that time

Table IX —Apparent Energetic Efficiency (Embryo)

Interdiurnal period	Increments of chemical energy (gm cals.)				A E E (embryo)
	Storage embryo (Needham (1928))	Combustion : embryo (Needham (1928))	Combustion corrected for combustion due to membranes (Table V, col 3)	Absorption (col 2 + col 4).	
1– 2	-	-	-		—
2– 3	—		-	—	—
3– 4	17	22	-	—	—
4– 5	36	45	—	—	—
5– 6	61	77	—	—	—
6– 7	101	121	82 5	183 5	55 1
7– 8	160	180	124 5	284 5	56 5
8– 9	232	260	184	416	55 9
9–10	358	367	263	621	57 5
10–11	538	509	376	914	58 9
11–12	860	684	529	1389	62 0
12–13	1340	902	834	2174	61 8
13–14	2340	1160	991	3331	70 2
14–15	3510	1450	1275	4785	73 5
15–16	4240	1750	1573	5813	73 0
16–17	4600	2030	1855	6455	71 2
17–18	5000	2250	2102	7102	70 5
18–19	5200	2400	2280	7480	69 5
19–20	5600	2530	—	—	—

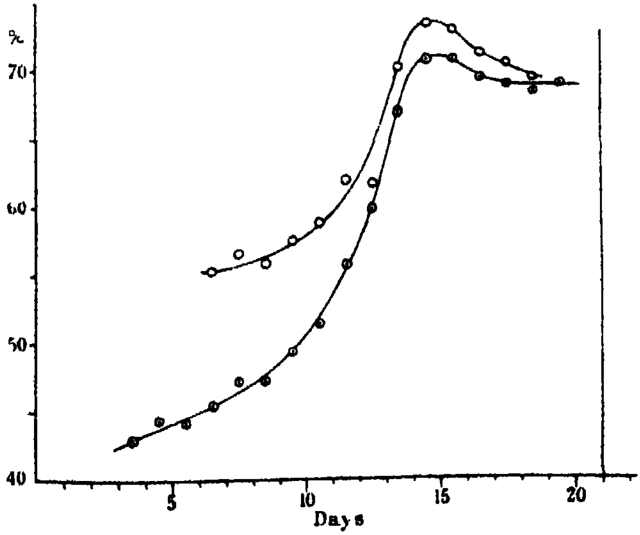

Fig 12 —Apparent Energetic Efficiency

● old curve ,

○ curve corrected for membranes.

Summary

(1) The respiratory intensity of the extra-embryonic membranes in the developing hen's egg, and the course of its change with time, has been investigated by manometric technique

(2) The yolk-sac maintains a steady level of respiratory rate at 320 c mm / gm /hr from the third to the fifteenth day of development, after which point it falls off, and has suffered a diminution of about 40 per cent by the time of hatching

(3) The respiratory rate of the allantois begins at a level of less than 100 c mm /gm /hr on the sixth day, then rises steadily to reach a maximum of 390 c mm /gm /hr on the thirteenth day, and subsequently falls to about 250 c mm /gm /hr by the time of hatching

(4) These points of inflection are synchronous with the changes occurring in the growth of the membranes The yolk-sac grows continuously from the beginning of development until the sixteenth day, after which it shows decreases in weight until hatching Thus its respiratory rate is steady during the period of its growth, but falls off as soon as it ceases to grow Similarly, the allantois, between the fifth and eleventh days, grows more rapidly than any other constituent of the living system, but after that point it remains at an approximately constant weight, having occupied the maximum space available for it within the egg And its respiratory rate increases continually as long as it is growing, but begins to decrease when its growth has stopped

(5) The percentage participation of the extra-embryonic membranes in the respiration of the intact egg has been calculated and found to decrease in a sigmoid curve from 32 per cent at the fourth day to 4 per cent at the nineteenth But the actual amounts of oxygen utilised by the membranes naturally follow their growth, and their summed respiration shows a maximum on the fourteenth day

(6) By subtracting the oxygen-uptake of the membranes from the oxygen-uptake of the intact egg (identical breed and flock), a more precise measure of the metabolic rate of embryo can be obtained than that derived from the classical practice of neglecting the respiration of the membranes The true metabolic rate of the chick embryo falls from 1200 c mm./gm /hr on the sixth day to 800 c mm /gm /hr on the nineteenth

(7) From a short discussion of the existing data on metabolic rate in embryonic life, it is concluded that a maximum of metabolic rate must exist during the development of the embryo of the chick. The results reported in this paper show that this must occur some time before the sixth day

(8) The Plastic Efficiency Coefficient of the developing hen's egg has been recalculated in the light of this research on the respiration of the membranes On the sixth day some 75 per cent of the material absorbed by the embryo is transmuted into living cells, by the ninth day this figure has dropped to 66 per cent, but by the end of development it has risen again to 78 per cent The efficiency of the membranes is much higher than this, indeed, remarkably high, thus, on the seventh day no less than 95 per cent of the material absorbed by the yolk-sac is turned into live weight, but this level is not kept up, and the figure has dropped to 72 per cent by the fourteenth day, after which it rapidly becomes nil owing to the cessation of growth—but not of respiration—on the part of the membranes The day by day efficiency of embryo and membranes together is the sum of these two sets of data, very high in the early stages, since the membranes are then ponderally the most important part of the living system, lower in the late stages, corresponding with the predominating weight of the embryo

(9) In the absence of information concerning the calorific value of the membranes themselves and of the material being combusted by them, the Apparent Energetic Efficiency could not be calculated so completely But the already existing curve for the embryo has been corrected on the interim assumption that at any given moment the solids combusted by the membranes have a content of chemical energy equivalent to those being combusted by the embryo

The author records with pleasure the generosity of Dr T C Byerly, who placed his extensive data for the growth and growth-rates of the extra-embryonic membranes in the hen's egg unreservedly at the service of this research. He is also indebted to Dr M Dixon for his kind co-operation in the use of the Warburg manometer, and to Dr D M Needham, who collaborated in certain of the experiments The expenses of this work have been partially defrayed by a grant from the Government Grant Committee of the Royal Society

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A Pigment Present in the Sweat and Urine of Certain Sheep. Its Isolation, Properties and Relationship to Bilirubin and the Metabolism of Hæmoglobin generally.

By CLAUDE RIMINGTON and A. M. STEWART.

(From the Wool Industries Research Association, Leeds.)

(Communicated by Sir F. Gowland Hopkins, P.R.S.—Received October 31, 1931.)

There is present in varying intensities in excretions surrounding the wool fibres of many sheep a golden brown colouration. Disposition to colouration appears to be an hereditary individual characteristic, but the depth of colour is influenced by conditions conducive to sweating, by diet and to some extent by age.

No reference to the cause of this colouration, the nature of the responsible pigment, nor to genetical work on predisposition to it can be found in the literature.

A previous paper (Stewart and Rimington, 1931) dealt with the economic aspect of its occurrence. Further work has resulted in the isolation of the responsible pigment from the wool yolk and examination of its properties. With the isolation of the same pigment from urine together with three others of a similar nature, confirmation of the opinion expressed in the previous paper, that the pigment was excreted in the sweat, has been obtained and its relation to the pigments of bile established. The resemblance between disposition to "golden colouration" in sheep, and familial acholuric jaundice in man is so marked as to warrant discussion.

Presence of the Pigment in the Water-soluble Fraction of the Yolk.

Raw wool consists of true wool fibres, associated with dirt, sand, etc., and about 40 per cent. by weight of "yolk." The yolk comprises the ether-soluble wool grease (15 to 35 per cent. by weight) and the "suint," water-soluble material, amounting to 5 to 20 per cent. of the total weight of the raw sample. In general, it may be said that the grease originates from the sebaceous glands and the suint from the sudoriferous glands of the animal. Wide variations are found to occur in the proportions in which these two constituents are present and naturally any conditions conducive to excessive sweating will increase the quantity of the latter.

It was found that the pigment of golden colouration is completely removed by normal soap-soda scouring. When, however, separate determinations of grease and suint were carried out the entire pigment was retained in the hot water extract. Closer examination of the suint fraction revealed a correlation existing between the depth of the colouration and the percentage of water-soluble solids, as shown in the following table:—

Australian Samples.

Sample	Appearance	Percentage of raw wool.	
		Grease	Suint
B	Bright, white fleece	22·0 22·3	1·2 3·3
D	Bright wool, greenish tinge, heavy condition	35·2 34·4	3·0 1·9
A	Creamy coloured, stylish wool, slight golden colouration	14·8 15·5	8·2 5·7
C	Yellow brown, intense golden colouration	25·4 27·9	12·8 15·4
S	Short, heavy, unattractive South African wool, orange-brown	25·5 24·2	15·0 12·6

Potchefstroom Samples (South Africa).

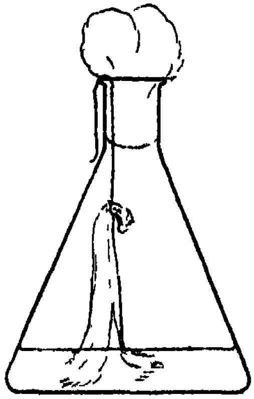
Sample	Appearance	Percentage of raw wool.	
		Grease	Suint
H	Less condition, more golden colour, yellow-brown	24·4 21·4	9·6 9·8
I	More condition, less colour, fairly bright	31·8 31·8	3·5 2·9
E	Wool from inside of neck fold, yellow-brown	37·5 36·8	8·1 8·0
G	Wool from crest of neck fold, same sheep, fairly bright	30·3 26·0	6·0 5·4

This correspondence between depth of colouration and amount of suint led to the hypothesis that the pigment is a constituent of the secretion of the sudoriferous glands, and is not to be associated with the wool grease

A review of the possible causes of this colouration was now made with a view to confirming or disproving the hypothesis Possible causative agencies may be classified as follows —

(1) Secretion of a pigment by either sebaceous or sudoriferous glands
(2) Secretion of a chromogen or precusor which is transformed into pigment by the activity of micro-organisms residing upon the wool
(3) Elaboration of the pigment by specific parasitic micro-organisms which deposit it upon the fibre

Experiments were undertaken to test the second and third possibilities Burgess (1925) has shown that the formation of pyocyanin by *B pyocyaneous* growing on wool can be obtained upon samples originally colourless when these are incubated in contact with infected wool under suitable conditions. Portions of raw, golden coloured Merino wool from Australia or South Africa were twined with *normal, white wool, the sample of the latter being in some cases* greasy, in others scoured, and the lock thus formed was suspended in a stoppered Erlenmeyer flask so that the tip entered a layer of water at the bottom of the flask (see diagram) *



There was no development of colour after incubating at 25° and 37° C

Extracts of golden coloured wool were made by shaking with sterile saline solution Samples of wool were incubated after soaking in the extracts, but with uniformly negative results

On plating out the saline extracts upon agar, numerous colonies were obtained, some of which were picked off and developed in pure culture from which extracts were made and tested as above, but without result

In order to determine whether the golden pigment could be derived from the action of micro-organisms inhabiting the wool upon some substance present in the sweat of only those sheep which develop colour under natural conditions, samples of wool were taken from the inside and from the crest of the neck fold of a South African Merino fleece which exhibited golden colouration in a fairly marked degree The wool from the crest of the fold was much lighter in colour, corresponding to the lower content of suint than that normally within the fold

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* We are indebted to Mr Burgess for assistance in carrying out the bacteriological experiments and also for permission to reproduce this diagram illustrating his technique developed for use with wool samples.

These samples, it was reasoned, should contain any precursor that might be essential for the development of the colour When incubated with coloured wool or saline extracts under conditions similar to those described, no indication of colour formation was observed

It was concluded therefore that micro-organisms play no part in the causation of golden colouration, and since relationship with the sebaceous glands has been excluded, there remains only the possibility that the pigment is secreted with the sweat, a hypothesis to which none of the known facts is contradictory

Isolation of the Pigment responsible for Golden Colouration

It was hoped that isolation and examination of the pigment would yield further evidence as to its nature and origin

As raw material for the isolation, 12 lb of a fleece of a 2-year old Australian Merino ram was selected showing fairly intense golden-brown colouration The previous history of the animal was known in detail In the isolation of the pigment, the desire to avoid any treatment likely to alter its nature resulted in the adoption of the following method

Ether-soluble material was removed by extraction in a large Soxhlet apparatus, portions of about 1½ lb being used at a time in the container The ether solution of the grease was of a pale straw colour

After degreasing, the wool was steeped in three changes of warm water, the washings being combined and centrifuged at a high speed. Even so, much mud and foreign material was left in colloidal suspension

Attempts to deposit the colloidal matter, and concentrate the solution by freezing, having proved impracticable on a large scale, one-half volume of 97 per cent alcohol was added to the unfiltered extract, and the p_H adjusted to 3·5 by addition of sulphuric acid, and enough solid ammonium sulphate added to cause the separation of the liquid into two layers as in Garrod and Hopkins' (1899) process for the isolation of urobilin The upper alcohol layer contained nearly all the pigment, whilst the mud collected in a compact layer at the interface. An acidity corresponding to a p_H of less than 4 0 was required to induce the majority of the pigment to pass into the upper layer. Ammonium chloride, instead of ammonium sulphate, did not satisfactorily effect the separation

The alcohol layer was removed and treated with an equal volume of ether. Again separation into two phases occurred, the lower aqueous phase containing the pigment, now much concentrated, whilst in the ether (pale yellow in colour) were retained the fatty acids, caproic, caprylic, etc., present as their salts in sheeps' suint (Buisine, 1886). Owing to their high emulsifying power, these substances, if not removed, were found to interfere seriously with the subsequent isolation of the pigment. It would appear that the pigment which, when pure, is insoluble in water, is retained in the suint as a result of the dispersive power of the soaps of these fatty acids.

After a second ether extraction, the aqueous solution, now a deep mahogany red colour, was neutralised with ammonia and slightly concentrated under reduced pressure to remove ether and alcohol. It was then re-acidified to the necessary p_H, 3·5, and solid ammonium sulphate added in the proportion of 28 g. crystalline salt per 100 c.c. of original liquid. This is sufficient to precipitate the pigment as a dark and somewhat sticky mass, whilst leaving in solution a colourless substance which exhibits great viscosity when in watery solution.

Reprecipitation with ammonium sulphate in the same proportions was repeated three or four times, after which the pigment was dissolved in a small quantity of water and precipitated by addition of the minimum amount (about 3½ to 4 volumes) of 97 per cent. alcohol. On stirring up with water, some tendency to viscosity was noticed, but after repetition of the alcohol precipitation all trace of this was absent. The pigment was finally stirred up with a small quantity of water and dialysed to remove diffusible salts until sulphate-free.

The resulting solution, which had a p_H of 4·0, was obviously only a colloidal suspension, since in addition to being opaque, part of the dispersed material was deposited on centrifuging. Addition of ammonia resulted in a clear dark brown solution which, on re-acidification to p_H 3·5, once more gave a colloidal suspension.

Some of the material was precipitated at this stage by four volumes of alcohol, dehydrated and analysed, with the following results:

*Analysis.**

	C	H	N	O	
	Per cent.	per cent.	per cent.	per cent.	(by difference)
Preparation 1	49·37	6·17	9·89	34·57	Ash 1·85 per cent.
„ 2	51·59	5·87	11·14	31·40	Ash 5·23 per cent.

* Micro-analysis by Dr. A. Schoeller, Berlin.

Although the material was still somewhat impure, the high proportion of oxygen appeared to be worthy of note Sulphur was tested for but found to be absent The ash, which contained a trace of iron too small to be accurately determined (less than 0 03 per cent), consisted mainly of silica.

The following tests were carried out —

Solubility —Insoluble in organic liquids, very slightly soluble in 50 per cent aqueous alcohol Forms colloidal dispersions in water the p_H of which is approximately 4 0 Dissolves readily to clear brown solution in dilute alkali, or sodium carbonate

Absorption —The solution exhibits no characteristic absorption band, neither could any be observed at any stage in the isolation There is general absorption of the violet and blue end of the spectrum

Reducing Agents —No change was observed on adding either sodium sulphite or hydrosulphite to acid or alkaline solutions of the pigment

Oxidising Agents —Hydrogen peroxide (10 per cent) or nitric acid changed the colour gradually to a clear, pale yellow Potassium permanganate is discoloured by an acid solution of the pigment and very slowly changes the colour of the latter to pale yellow Aspirating air through an alkaline solution was without effect

Biuret Test —Negative

Millon —Negative

Xanthoproteic —Possibly a feeble reaction

Diazo Reaction (*sulphanilic acid*) —Negative whether in acid or alkaline solution

Zinc Acetate Test —Negative

Iodine —Negative

Molisch —Faint, but positive This test was given persistently by the pigment throughout the stages of purification, but it was suspected that it may have been due to a carbohydrate impurity of colloidal character

Fehling's Solution —Negative Feebly positive after boiling with acids

Pine Splinter Reaction —Positive, intense.

The most characteristic feature emerging from the above observations was the comparative stability and chemical inertness of this pigment It has been named "Lanaurin," indicative of its origin from golden coloured wool.

Some of the crude material was boiled gently with 5 per cent. sulphuric acid for about 3 hours. On centrifuging the resultant mixture, practically the entire pigment was obtained as a dark amorphous deposit, whilst the supernatant liquid contained some reducing substance, possibly sugar, and a little ammonia.

After repeated washing with water, the pigment was dissolved in dilute ammonia and reprecipitated by sulphuric acid, washed again with water and finally dehydrated by alcohol and ether. It differed from the crude material in giving a negative Molisch reaction and in no longer forming colloidal suspensions when ground up with water. Otherwise it appeared to be physically unchanged. The mean analytical figures calculated for the ash-free substance agreed satisfactorily with an empirical formula $C_{33}H_{36}N_4O_{10}$.

Analysis.

	C	H	N	O	
	per cent.	per cent.	per cent.	per cent.	(by difference)
	59·92	5·63	8·26	26·19	Ash 1·58 per cent.
$C_{33}H_{36}N_4O_{10}$ requires	61·1	5·5	8·6	24·7	

In appearance the pigment is a dark, brownish-black, amorphous substance, decomposing without melting. It gives an intense pine splinter reaction but a negative Lassaigne test for nitrogen in spite of containing 8 per cent. of this element. A similar failure of the Lassaigne test was observed by Lemberg (1929) working with a pyrrolic pigment from phycocyanine, and appears to be frequent in the case of pyrrole derivatives (Feist, 1902). Upon distillation of the pigment with zinc dust, no formation of indole was observed.

An attempt to form a methyl ester from a small quantity of lanaurin was unsuccessful since only unchanged material could be recovered at the conclusion of the experiment. About 0·25 g. of crude lanaurin was suspended in 25 c.c. of dry methyl alcohol into which hydrochloric acid gas was passed for 12 hours. The pigment gradually dispersed in the acid alcohol, but no colour change was observed. After slight dilution with water, an equal volume of chloroform was added but failed to extract any colouring matter. The upper solution was now shaken with ether, whereupon two phases appeared, the aqueous being the more deeply coloured. After separation and evaporation, a little water was added and the minimal quantity of ammonia necessary to dissolve the pigment. Acidification now precipitated the pigment in an easily centrifugeable form.

It was washed with alcohol, dehydrated and analysed.

The supernatant liquid derived from the original aqueous phase yielded, upon evaporation, some alcohol-soluble crystals These gave an intensely positive Molisch reaction and therefore most probably represented material originally present in the colloidal impurity already referred to

Analysis

Pigment recovered after attempted esterification —

C per cent	H per cent	N per cent	O per cent (by difference)	
53 43	5 52	9 25	31 80	Ash 4 36 per cent

Lanaurin is characterised by great resistance to reduction even with vigorous reagents, in this respect showing resemblance to bilirubin

To 0 2 g pigment was added 2 c c of hydriodic acid (S G 1 96) and 4 c.c of glacial acetic acid and the mixture kept for 24 hours in a vigorously boiling water bath After cooling, excess iodine was removed by phosphonium iodide and the solution filtered A large quantity of insoluble material closely resembling the pigment was left upon the paper The filtrate had a light yellow colour It was made alkaline with sodium hydroxide and shaken with ether, then acidified (to congo red) and repeatedly extracted with fresh quantities of ether The extracts were evaporated, separately, to dryness The former, which would have contained bases, left only a trace of residue From the latter, however, in which pyrrole acids were to be expected, a small amount of amorphous material separated This was dissolved in a small quantity of water and treated with picric acid A few milligrams of a micro-crystalline picrate were obtained which melted at 152–154° Pure iso-hæmopyrrole-carboxylic acid picrate melts at 160°

Isolation of the Pigment Lanaurin from Urine, together with other Pigments of Biliary Nature.

Proceeding upon the assumption that the pigment is a product of metabolism, the urine was next examined By submitting samples of urine from sheep exhibiting golden colouration to a similar method of isolation the same pigment was obtained as was found in the wool yolk.

In addition, other pigments were isolated which appear to be more closely related to those of bile than is lanaurin. Together, they comprise a series which may well represent stages of progressive change in which the solubility in organic solvents and power to give positive diazo reactions and coloured oxidation products is gradually lost and properties more nearly approaching those of the melanins acquired.

In appearance the samples of urine from the (Australian Merino) golden coloured sheep were rather dark in colour and brownish No absorption bands were detected and no marked fluorescence with zinc chloride, but the diazo reaction with diazotised sulphanilic acid in alkaline solution was strongly positive. There appeared to be also a feeble Gmelin reaction The capacity for the diazo reaction was found ultimately to reside in a reddish, chloroform-soluble pigment which also gave a somewhat atypical Gmelin reaction In particular, the bright green ring was reduced to a faint greenish nuance, whilst in the upper part of the colour series a strong, cherry red predominated

The isolation and separation of these pigments from the urine was accomplished as follows —

Approximately 1 litre of urine from a 2-year old wether, which exhibited golden colouration, was brought to p_H 3 5 by addition of 2N sulphuric acid One-half volume of alcohol was then added and sufficient solid ammonium sulphate to cause separation into two layers The upper layer, carrying almost all the colour, was treated with an equal volume of ether, and the lower aqueous phase separated This solution was neutralised with ammonia and concentrated on the water bath to remove ether and alcohol It was dark mahogany brown in colour and exhibited a faint green fluorescence

After filtering off a small residue, sulphuric acid was added to p_H 3 5 and then sufficient ammonium sulphate to precipitate the pigment It was centrifuged down, washed thoroughly with distilled water and then dissolved by the addition of the minimal quantity of dilute ammonia From the solution it was again precipitated by acidification, washed and finally dehydrated with alcohol and ether Only a very slight tendency to go into colloidal solution was noted and all washings, when coloured, were collected separately and worked up again by addition of a small quantity of acid which effectively flocculated the colloidal suspension The yield was 17 mg of pigment from a litre of urine

In general properties, this pigment exactly resembled lanaurin isolated from golden coloured wool It exhibited no absorption bands, only a general shading off in the blue-violet region of the spectrum It was insoluble in water, alcohol, or any organic solvent, but soluble in dilute alkali, giving reddish-brown solutions from which it could be completely precipitated by addition of acid to about p_H 3·5–4·0 It gave neither Gmelin nor diazo reaction, but the pine splinter test was positive. Analysis confirmed its probable identity with lanaurin

Analysis

	C per cent	H per cent	N per cent	O per cent (by difference)	
From urine—					
No 36	52 85	5 05	8 29	33 81	Ash 5 0 per cent
,, 46	52 73	5 34	8·89	33 04	Ash 4 1 per cent

The ether solution, obtained as a result of adding ether to the alcohol-ammonium sulphate phase in the second stage of isolation of lanaurin from urine was coloured a pale yellow-brown In order to recover the pigments it contained, it was evaporated to dryness upon the water bath The black, tarry residue was treated with chloroform which extracted a red-coloured pigment together with some colourless material characterised by a most disagreeable and penetrating smell Petroleum ether was found to remove this substance without dissolving the pigment, so that a separation was easily effected by thoroughly extracting the dry residue remaining when the chloroform was distilled off The colouring matter was again dissolved in chloroform, precipitated by addition of petroleum ether and finally deposited by the slow evaporation of a chloroform solution

The yield of this pigment was only 7 5 mg per litre of urine It was obtained in micro-crystalline form, but could only be crystallised with difficulty

The analytical results were as follows —

Analysis

Chloroform soluble pigment

	C per cent	H per cent	
Urine pigment	68·60	6 68	Ash 1·14 per cent
Bilirubin	67·81	6·21	

(The small quantity prevented nitrogen determination being carried out)

This pigment was readily soluble in alcohol, ether, chloroform and acetone, giving rise to a red solution showing no characteristic absorption bands It was insoluble in petroleum ether and in water It was dissolved by dilute aqueous alkalis yielding a reddish brown solution from which it could be precipitated by acidification.

The diazo test was strongly positive (red), the Gmelin test positive but hardly typical. Green was very faintly represented in the colour series whilst the upper band of cherry red was intense.

Lack of material prevented further examination, but it can hardly be doubted that this pigment stands in close relationship to bilirubin.

The residue of the original ether complex remaining, after chloroform had been used to extract the above pigment, was taken up in absolute alcohol, which dissolved all colouring matter. The intensely dark solution was filtered to remove ammonium sulphate and evaporated to dryness. This residue was suspended in water and a slight excess of dilute ammonia added. After filtration from insoluble material, the pigment was precipitated by acidification. This treatment was repeated several times the pigment finally being precipitated by ether from concentrated alcohol solution, dehydrated and analysed. The yield was 34 mg. per litre of urine.

Analysis

Alcohol soluble pigment

C	H	N	O	
per cent	per cent	per cent	per cent	(by difference)
59.41	5.28	4.60	30.31	Ash 1.67 per cent

In general properties (apart from the lower nitrogen content, similar to the 4.1 per cent. of nitrogen found by Garrod and Hopkins (1897) in urobilin) this pigment appeared to stand midway between that soluble in chloroform and lanaurin, which is soluble only in aqueous alkali.

Although readily soluble in alcohol, it was found to be insoluble in all other organic solvents tried. Like the other two it could be dissolved in aqueous alkalis and reprecipitated by addition of acid. Both diazo test and Gmelin reaction were negative and no characteristic absorption spectrum could be observed.

It would appear that the three pigments isolated from these urines represent stages in the gradual modification of a pyrrolic complex derived ultimately from haemoglobin and suffering progressive change in the direction of a product more nearly allied to the melanins than to the pigment of the bile.

In a recent review of our knowledge of the constitution of porphyrins and related pigments, Fischer (1930) points out that only so long as the four centred pyrrole rings are intact is a characteristic spectrum with absorption bands exhibited When the ring system becomes opened out to a chain this property is lost, and when further alteration take place in the structure of this chain the capacity to give the Gmelin reaction simultaneously disappears

That the same pigment should be found in the sweat and urine of sheep exhibiting golden colouration is not surprising when the close similarity is borne in mind between the renal system and the sudoriferous glands, and the products of their activity A comparison of the composition of urine and of sweat respectively, justifies one in regarding the sudoriferous system in some respects as an auxiliary kidney The accompanying table has been constructed from average values, and to facilitate comparison all sweat figures have been multiplied by four since it would appear that sweat is considerably more dilute than is urine

	NH_3 — N, mg /100 c c	Urea — N, mg /100 c c	Amino acid — N mg /100 c c	Chlorides, *g* NaCl/litre
Sweat × 4	42	84	16	12
Urine	40	120	12	10

Palmer and Cooledge (1914) have shown conclusively that the yellow pigment normally occurring in milk whey is identical with urochrome, the typical urinary pigment, and it is also of interest to note that small quantities of a pigment were encountered by them, which differed from urochrome, in being precipitated by saturation with ammonium sulphate It is possible that this pigment was lanaurin or closely related to it. They also found that the whey of sheep's milk contained up to four times as much pigment as is normally present in cow's milk It is quite probable, therefore, that lanaurin could also be isolated from the milk of sheep showing golden colouration.

Differences in permeability of renal and other cell membranes in different animal species have been observed Saiki (1930) found that dogs' kidneys are more readily permeable to bilirubin than are those of rabbits.

The urine from a number of sheep regarded as normal was collected at the slaughter house On examining it by the same method, it was found, although the urine was quite darkly coloured practically the whole of the pigment, on shaking the alcoholic extract with ether, went into the ether-alcohol layer. There appeared to be no pigment present that could be precipitated by ammonium sulphate from the aqueous, underneath layer

The solvents were evaporated off and the tarry residue was extracted with chloroform which removed some of the pigment as a reddish solution.

In this normal urine, therefore, three of the four pigments found in the other urines were present—lanaurin appeared to be absent.

From a sample of urine* from a sheep known to be affected slightly with liver rot, lanaurin was isolated without difficulty.

Possible Similarity between Golden Colouration in Sheep and Familial Acholuric Jaundice in Man

Chromidrosis, or the secretion of coloured sweat, is clearly recognised in medical practice, although opinions differ as to the extent of the part played by bacteria in its causation Some cases undoubtedly are traceable to bacterial origin, true chromidrosis, the secretion of a pigment by the sudoriferous glands, has been described by Gans (1905), Foot (1869, 1873), Mitchell (1899), White (1884), and others

In Gans' case the pigment excreted was indican, in that recorded by Mitchell it was a substance insoluble in ether, but soluble in acid aqueous solutions, bacteriological examination failed to throw any light upon its occurrence It is perhaps noteworthy that in nearly all cases of chromidrosis, some degree of chronic intestinal stasis was complained of by the patient This might well lead to the circulation in the blood of abnormal pigments

Vass and McSwiney (1930) state that from all the samples of human sweat examined by them pigment was absent and that the sweat was "almost colourless" It is, however, questionable whether in such dilution as obtains in normal sweat the presence of any pigment would be recognised, especially since the material is generally clouded by cell debris In the case of sheep's wool, a 12-months' fleece contains the accumulated solids of a quantity of upwards of 50 gallons of sweat The enormous concentration would render evident small quantities of pigment barely noticeable in freshly shed perspiration The total yield of pigment, making allowances for manipulation losses, was probably not more than 5 g from the 12 lb of golden coloured wool used in the experiment detailed above It is not improbable, therefore, that human sweat may actually contain pigment substances not clearly evident under normal conditions

Possible Relationship between Golden Colouration and Acholuric Jaundice

It is natural to assume that lanaurin, the pigment of golden coloured wool, is derived ultimately from hæmoglobin set free from broken down erythrocytes

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* We are indebted to Dr Montgomery (Bangor) for this sample

The suggestion is supported by the chemical composition of the pigment, and by its undoubted pyrrolic nature Lanaurin differs from bilirubin and urobilin in its insolubility in organic solvents. It would appear to be a partially condensed system, representing a stage between the simple tetrapyrrolic nucleus and a fully condensed melanin

The metabolism of hæmoglobin is but imperfectly understood Within recent years it has become customary to assign chief importance in this matter to the cells of the reticulo-endothelial system (Aschoff, 1922) such as the Kupfer cells of the liver and those of the spleen Their action is visualised as destructive, being exerted upon (possibly effete) erythrocytes of the blood The pigment liberated within these cells is transformed into bile pigment, but little is known concerning the details of this process (see Rich, 1925), or of the series of changes by which bilirubin is later transformed into urobilin

On the basis of a colour reaction with diazotised sulphanilic acid, Van den Bergh (1918) was led to postulate two varieties of bilirubin, and this distinction served him as a basis for the differentiation of the various clinical types of jaundice More recent work by Griffiths and Kay (1930) would suggest, however, that two entirely different pigments are involved in the direct and indirect Van den Bergh tests, one of which is insoluble in chloroform, but soluble in alcohol or alkaline aqueous media It is conceivable that pigments of the latter type may eventually find their way into the secretions of sudoriferous glands in cases where pigment metabolism is proceeding upon an unusually high level That this may be the case even in normal sheep is suggested by the high pigmentation of the milk of these animals as recorded by Palmer and Cooledge

Acholuric familial jaundice seems to have been first described by Wilson (1890), who correctly diagnosed its origin in splenic hyperactivity, quite soon afterwards the disease was independently recognised in France and in Germany. Hayem (1897, 1899) showed that whilst rich in urobilin the urine contains no bilirubin, although this pigment is present in abnormally large quantity in the blood Minkowski (1900), describing a family in which there were eight cases in three generations, added the fact that, post-mortem, the kidney is found to be rich in iron

The peculiar hereditary character of the disease has attracted much attention, and it has been traced in several families through successive generations. Meulengracht (1922) gives an account of eight families including 33 subjects with acholuric jaundice In the families with one parent affected there were 19 children with the disease and 13 who were healthy, or, if a few doubtful cases be included, the numbers become 26 and 24, which is in surprisingly good agreement with the 1 1 ratio expected on the basis of Mendel's theory, if the character in question be regarded as a simple dominant

Campbell (1921), who described a case of familial jaundice in 1921, was led to a contradictory conclusion, however, in two later papers on the disease (1925) he described investigations made upon the fragility of the red corpuscles in individuals of the same family These studies again demonstrate the simple Mendelian character of the affliction, and bring to light the significant fact that in some persons the external signs may not be clearly noticeable although the diminished resistance of the erythrocytes to hæmolysis proclaims the presence of the disease It is well known that the effect of certain Mendelian dominant factors may, at times, remain latent, an appropriate set of external conditions being required to make them manifest (Hoge, Morgan, Garrod)

Whilst very little is accurately known concerning the inheritance of golden colouration in sheep, there are certain points which this abnormality exhibits in common with hereditary acholuric jaundice In the first place, the condition is obviously transmissable, but all degrees of intensity of colouration are met with, suggesting, possibly, that conditions may influence greatly the external signs Working with the urine of cattle Warth and Ayyar (1930) found that its acidity was considerably affected by the type of food ingested Urine from cattle fed on wheat straw was acid, on over-ripe hay acid or neutral, and on green fodders and other cereal straws alkaline If it may be assumed that sheep react in the same way to diet, it would be expected that food which would tend to make the urine acid would thereby tend to reduce the permeability to these alkali soluble pigments In practice it has been observed that sheep disposed to golden colouration grazing largely on wheat straw and dried grass do not develop much colour The pigment in the wool yolk is much more pronounced when green feed is available The pigment is of the biliary type and, if our hypothesis be correct, may be found to result from overactivity of the reticulo-endothelial system, including the spleen, with consequently enhanced red cell destruction Whilst partaking in this connection of the character of a disease, there is no reason why sheep exhibiting golden colouration should be regarded as inferior to normally coloured animals The condition is rather one of balance between a hyperactivity within physiological limits and a definitely pathological state (compare McNee, 1922–23).

It would be of interest to follow up these investigations by breeding experiments in which not only the external colour but also the size of the spleens and hæmolytic index of the animals from successive generations were determined

Relatively enhanced pigmentation of blood, urine and milk would also be expected in golden coloured animals should the arguments outlined above be true

We wish to express our thanks to Professor Haldane, Dr Keilin, Dr Lemberg and Dr Burton, for the interest which they have taken in this work and for many valuable suggestions We also wish to thank Professor McSwiney for granting us the use of the large-scale biochemical operation plant and for the generous hospitality extended to us whilst working for some days in his Department A M S, of the University of Western Australia, wishes to thank the Council of the Wool Industries Research Association for facilities granted him for working temporarily in their laboratories

Summary

(1) The phenomenon of golden colouration of the wool of certain sheep has been further investigated, and the hypothesis, put forward previously, that the colouration is due to a pigment excreted in the sweat, has been confirmed

(2) This pigment, termed *lanaurin*, has been isolated from golden coloured wool and appears to be a pyrrolic complex It probably occupies an intermediate position between the bile pigments and fully condensed melanin.

(3) The same pigment has been isolated from the urine of sheep exhibiting golden colouration

(4) Accompanying lanaurin, in such urines, other pigments were found, more closely similar in properties to bilirubin These have been isolated and investigated

(5) Attention is directed to the similarity between golden colouration in sheep and familial acholuric jaundice in man. Both conditions are inherited, factor responsible behaving as a simple Mendelian dominant, in the case of the human disease, and most probably also in the case of sheep, although in this latter connection much less evidence is at present available.

(6) As a result of the present investigation the general conclusion is drawn that the two phenomena are similar and that golden colouration in sheep is to be attributed to an hereditary tendency to hyperactivity of the cells of the reticulo-endothelial system, with a consequently enhanced destruction of hæmoglobin The pigment lanaurin is considered to be derived ultimately from hæmoglobin and to be excreted from the circulation via the renal and sudorific systems

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The Functional Activity of the Vasomotor Nerves to the Lungs in the Dog

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Owing to the large amount of conflicting data regarding the activity of the pulmonary vasomotor nerves, modern reviewers of the literature generally conclude that vasomotor influences play no important part in the regulation of the pulmonary blood flow Previous work on the subject falls sharply into two groups, experiments on the whole animal and those on isolated perfused lungs It must be confessed that a careful survey of the work on the whole animal purporting to demonstrate the functional activity of these nerves reveals with few exceptions the absence of a sufficient proof owing to the difficulties of obtaining well controlled experimental conditions, and this no doubt accounts for the scepticism prevalent at the present time Among the exceptions are the classical experiments of Bradford and Dean (1889, *a*, 1889, *b*, 1894) on the dog, curarised and atropinised These investigators showed that stimulation of the peripheral ends of the cut thoracic nerves from D2-D7 would sometimes cause a pulmonary arterial pressure rise without either cardiac acceleration or a systemic arterial pressure rise, and in general their results were confirmed by François-Franck (1895, *a*, 1895, *b*) and Plumier (1904, *a*) Excitation of the vagus in these experiments produced no effect on the pulmonary arterial pressure, probably because, as suggested by Luckhardt and Carlson (1921), atropine was injected to eliminate the effect of vagus stimulation on the heart Further, they presented evidence of reflex pulmonary vasoconstriction caused by excitation of the upper thoracic posterior roots, and François-Franck (1896) was able to demonstrate the same effect by stimulation of the central ends of the splanchnic or anterior crural nerves Bradford and Dean (*loc cit*) and Weber (1910) also observed that a reflex change in the pulmonary arterial pressures occurred after central stimulation of the divided vagus nerve, and Schafer (1920) reported a reflex pulmonary arterial pressure fall after stimulation of the depressor nerve The investigations of Bradford and Dean and of Wood (1902, 1910, 1911) on the relations of the pulmonary and aortic pressure during asphyxia were adduced as evidence in favour of the presence of a vasoconstriction nerve supply to the lungs, but the production of an adrenalinæmia in these experiments was not considered

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* Fellow of the Rockefeller Foundation.

If a change in the pulmonary arterial pressure or in pulmonary blood flow is to be taken as the criterion of the presence of functional vasomotor nerves to the lungs when a portion of the nervous system is stimulated, it is necessary in experimenting on whole animals to eliminate concomitant variations in systemic arterial pressure and capacity, and in cardiac rate and output which might influence the pulmonary arterial pressure or blood flow (Openchowski, 1882) Until 1889, as pointed out by Bradford and Dean, investigations which purported to demonstrate the activity of vasomotor nerves were not always controlled in this manner, although Henriques (1892) found that stimulation of the vagus in non-atropinised animals (dog and rabbit) produced a fall in pulmonary arterial pressure and a rise in left auricular pressure, and in the cat (one experiment) a rise in pulmonary arterial and a fall in left auricular pressure In the absence of a change in heart rate he concluded the vagus carried pulmonary vaso constrictor fibres in the cat and vasodilator fibres in the dog and rabbit. François-Franck (1880) (on dog) also obtained, on excitation of the thoracic sympathetic nerves to the lungs a pulmonary arterial pressure rise

In spite of these earlier results doubt was still cast upon the functional activity of the pulmonary vasomotor nerves, partly because in the whole animal experiments, one uncontrolled factor was the force of the beat of the ventricles which might be augmented on stimulating the sympathetic nerve supply to the lungs, although the heart rate might remain constant (Brodie and Dixon, 1904; Dixon and Hoyle, 1928), and partly because of the inability of some investigators to repeat these observations (Erikson, 1907, Romm, 1924) It should be mentioned that Brodie and Dixon were able to confirm most of the experimental results obtained by Bradford and Dean, but were unable to agree with them in concluding that the effects they observed could only be explained on the supposition that the pulmonary vessels were supplied with constrictor fibres and were therefore under nervous control

If we turn to the experiments on isolated perfused lungs, however, we find they prove conclusively that the pulmonary vessels are supplied with nerves, stimulation of which produce vasodilator and vasoconstrictor effects Plumier (on dog) (1904, *b*), Tribe (on cat) (1914), and Le Blanc and van Wijngaarden (on cat) (1924), observed vasoconstriction following stimulation of the sympathetic nerves, Cavazzani (on rabbit) (1891) the same effect after stimulation of the vagus Pulmonary vasodilatation after excitation of the vagus was obtained by Le Blanc and Wijngaarden (on cat) and after stimulation of the cervical sympathetic by Cavazzani (on rabbit) (1891) and by Tribe (on cat) (1914) after excitation of the stellate ganglia. Negative results were reported by Brodie and Dixon (on cat) (1904) and by Lohr (on cat) (1924)

Baehr and Pick (1913) were unable to obtain any pulmonary vasoconstriction after addition of adrenaline to the Tyrode solution perfusing guinea-pigs' lungs and on this evidence concluded that there is no basis for postulating vasoconstrictor endings in the lungs

According to Krogh (1907) pulmonary vasoconstrictor fibres are present in the vago-sympathetic of Testudo græca, and of Rana pipiens and turtle (Luckhardt and Carlson, 1921) Nisimaru (1922)* obtained pulmonary vasoconstriction on stimulation of the peripheral end of the vagus trunk in the neck (toad, tortoise) and found these fibres to be sympathetic in origin, arising from the 3rd and 4th spinal nerves to pass to the corresponding sympathetic ganglion, thence to the jugular ganglion and finally in the vagal trunk

After reviewing the experimental evidence there can be no doubt that the functional activity of the pulmonary vasomotor nerves has been definitely established in spite of statements to the contrary. It is true that in the more rigidly controlled perfusion experiments the alterations in pulmonary blood flow and pressure in response to nerve stimulations have not been very large, but this is in part due to the difficulties encountered in keeping all perfused preparations in good condition The point at issue appears to be not whether the pulmonary vasomotor nerves are functionally active but whether they are able to exert a powerful enough control to produce significant changes in the pulmonary blood flow and arterial pressure, and perhaps in the redistribution of blood within the lungs. Experience gained with isolated lungs in which the pulmonary and bronchial vascular systems are perfused simultaneously led Berry and Daly (1931) to believe that the functional activity of the vasomotor nerves would be greater in these preparations than in those perfused only through the pulmonary artery, especially as Berry, Brailsford and Daly (1931) showed that the larger intrapulmonary nerve trunks derived their blood supply to a large extent from the bronchial arteries They suggested that the small or absent vasomotor effects obtained by some workers might have been due to the cutting off of the blood supply to the nerves with consequent loss in their excitability and conductivity. We decided therefore to make an attempt to obtain fresh information using this preparation

II. METHODS

The lung-œsophagus or isolated lung preparations, with pulmonary and bronchial vascular beds perfused in the manner described by Berry and Daly (1931) are set up with the cervical or thoracic vagosympathetic trunks and stellate ganglia on both sides carefully exposed Shielded electrodes surrounded the nerve structures to be stimulated and the current from a Du Bois-Reymond induction coil (C F Palmer, Ltd) is used for excitation

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* In Japanese Dr Nisimaru kindly supplied a reprint and translation of the main conclusions.

These Palmer coils have approximately 300 turns of 20 S W G , D S copper wire in the primary, wound on an iron core ½ inch diameter The secondary coil consists of approximately 7500 turns of 38 S W G enamel copper wire and the normal frequency of the vibration ranges between 60 and 80 per second Two dry cells giving 2 3 volts on load produce a secondary current which is just bearable on the tongue with the coils 9 cm apart

Similar nerve structures on each side of the preparation are stimulated simultaneously and switching arrangements are provided for the rapid change of stimulation of one pair of nerve structures to the other Thus both cervical vagosympathetic nerves could be stimulated and then both stellate ganglia with only a short interval of time between the two operations

We have determined the effects of exciting the sympathetic and parasympathetic paths at the following levels —

(1) The cervical vagosympathetic (C V S) nerves 6–10 cm above the inferior cervical ganglion (I C G)

(2) The separated cervical vagi or sympathetic nerves at the same level.

(3) The thoracic vagosympathetic (T V S) nerves immediately below the inferior cervical ganglia The so-called thoracic vagus contains sympathetic fibres and should therefore be called thoracic vagosympathetic (Mollgaard, 1912 , Braeucker, 1926)

(4) The stellate ganglia (S G)

(5) The thoracic vagosympathetic nerves at the root of the lungs

The thoracic vagosympathetic trunks were generally prepared for stimulation after the cervical nerves had been tested In some experiments the sympathetic cardiac fibres from the stellate ganglia and limbs of the Ansa Vieussenii were left intact, in others destroyed

III. Experimental Investigations

1 *The Effect of Nerve Stimulations on the Pulmonary Arterial Pressure*

In each experiment the output of the two pumps supplying the pulmonary and bronchial vascular beds was not altered during the nerve stimulations so that a rise in pressure denotes a vasoconstriction, a fall, a vasodilatation

(i) *Cervical Vagosympathetic Nerves.*—Stimulation of these nerves in 12 preparations, caused pulmonary vasoconstriction in four, fig 1*b*, slight pulmonary vasodilatation in two, fig 2*a*, bronchoconstriction in five, and bronchodilatation following bronchoconstriction in one Five preparations gave negative results The slight pulmonary vasodilatation and bronchoconstriction

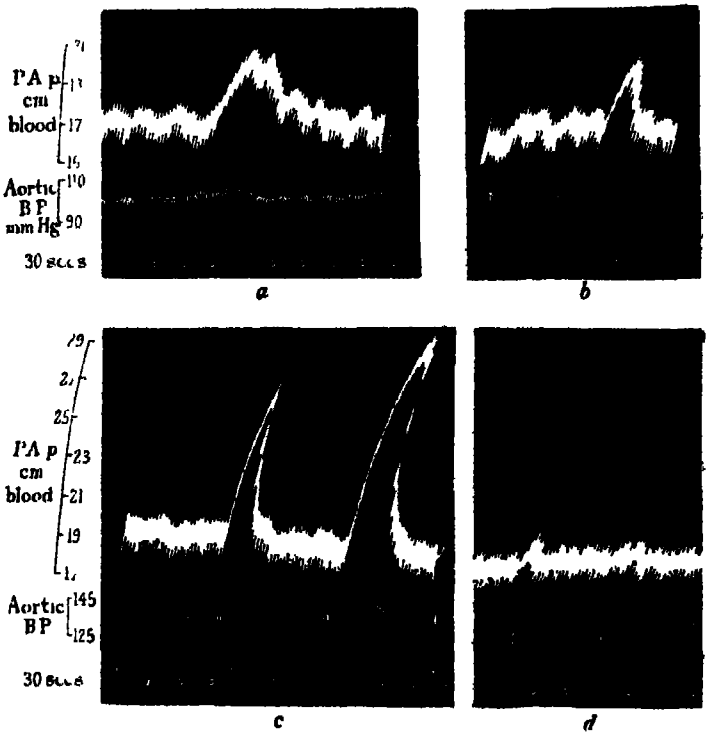

FIG 1 —Dog, 9 0 kg Lung œsophagus preparation Simultaneous perfusion of the aorta and pulmonary artery *a* Stimulation of both S G, coil distance — 11 cm *b* 8 minutes later, stimulation of both C V S, coil distance - 9 cm, 8 minutes after *b* " stimulation of both S G or both C V S with coil distance = 0 cm gave negative results Between "*b*" and "*c*" 2 0 mg atropine sulphate was injected into the aorta and 3 0 mg into the P A *c* Electrodes shifted to T V S below I C G Two successive stimulations of T V S 12 minutes after atropine, coil distance = 5 cm Between "*c*" and "*d*" 2 5 mg of ergotamine tartrate injected into P A and 1 5 mg into aorta *d* Two successive stimulations of both T V S 15 minutes after ergotamine, coil distances — 14 and 5 cm The tidal air was not affected by the nerve stimulations R.P = 0 to - 15 cm H_2O. S G = stellate ganglia C V S = cervical vago sympathetic nerves C V — cervical vagi T V S = thoracic vagosympathetic nerves I C G — inferior cervical ganglia P A = pulmonary artery R P = respiratory (intrathoracic) pressure

is removed by atropine, fig. 2 The pulmonary vasoconstrictor response which may occur in the presence or absence of bronchoconstriction has not been tested against atropine or ergotamine

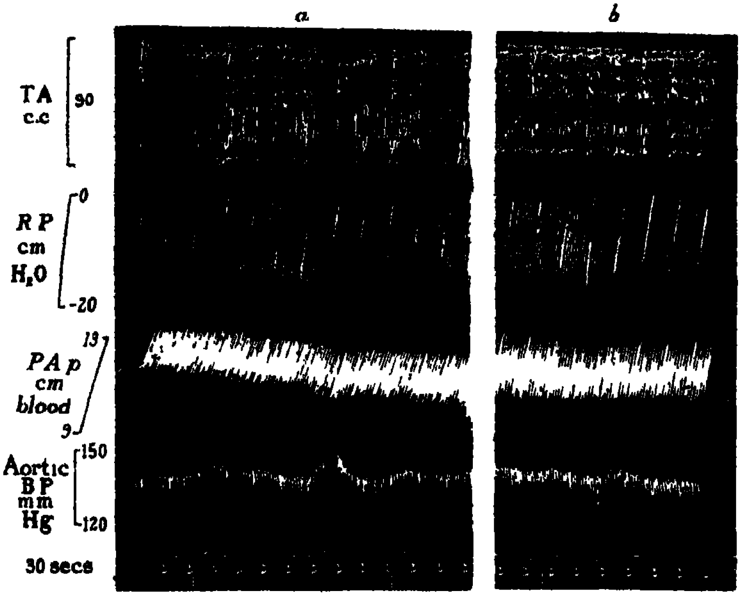

Fig 2.—Dog, 12 5 kg Lung-œsophagus preparation Simultaneous perfusion of the aorta and pulmonary artery *a* At 1, stimulation of both S G, coil distance = 0 cm, at 2, stimulation of both C V S, coil distance = 6 cm, at 3, stimulation of both C V S., coil distance = 3 cm, at 4, stimulation of both S G., coil distance = 0 cm Between "*a*" and "*b*," atropine sulphate 5 0 mg into P A and aorta injected *b* At 1, stimulation of both S G, coil distance = 0 cm, at 2, stimulation of both C V S., coil distance = 0 cm T A = tidal air

(ii) *Cervical Vagi.*—In three preparations tested, pulmonary vasoconstriction occurred in two, fig 3, *a*, very slight pulmonary vasodilatation in one, fig 4, bronchoconstriction in one and bronchodilatation following bronchoconstriction in one In one preparation tested cervical sympathetic nerve stimulation had no effect on the pulmonary vessels or bronchial tubes The effect of stimulation after atropine has not been tried

(iii) *Stellate Ganglia.*—Pulmonary vasoconstriction occurred in three, fig 1A, bronchoconstriction followed by slight bronchodilatation in one and slight bronchodilatation in one Ten preparations were tested and six were negative.

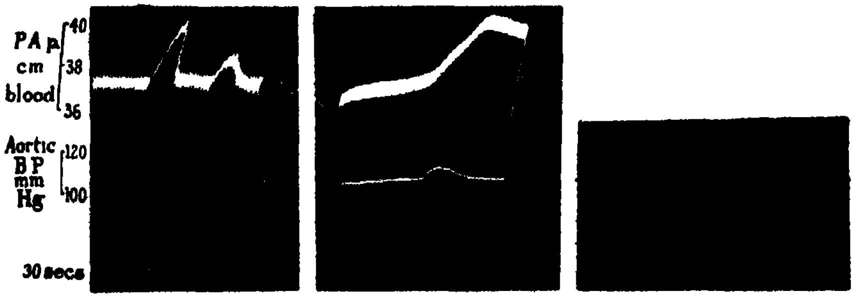

FIG 3.—Dog, 11 5 kg Lung-œsophagus preparation Simultaneous perfusion of aorta and pulmonary artery Length of œsophagus in circulation = 2 5 cm *a* Two successive stimulations of the C V which are separated from the C V S trunks, coil distance = 7 cm *b* Stimulation of T V.S trunk, 22 minutes later, coil distance = 7 cm At the height of the pulmonary arterial pressure rise, the perfusion pumps were stopped and the lungs removed, the trachea and œsophagus being left *c* Three minutes after "*b*" two successive stimulations of the T V S trunks caused a rise in aortic pressure. The blood flow from the azygos vein fell from 61 to 43 drops per minute during the nerve stimulation and recovered subsequently to 63 drops per minute

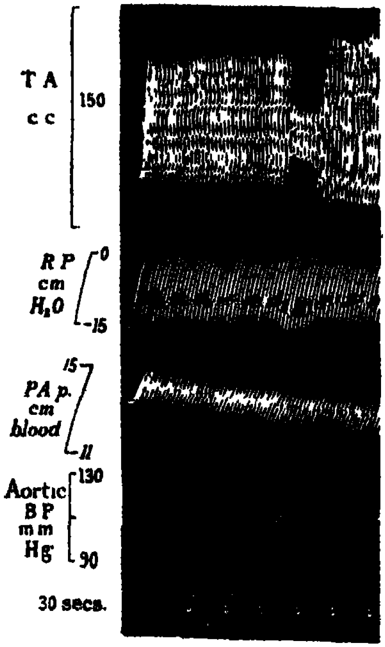

FIG. 4.—Dog, 12 0 kg Lung-œsophagus preparation Simultaneous perfusion of aorta and pulmonary artery. At 1, stimulation of both G.S, coil distance = 10 cm, at 2, stimulation of C.V., coil distance = 8 cm

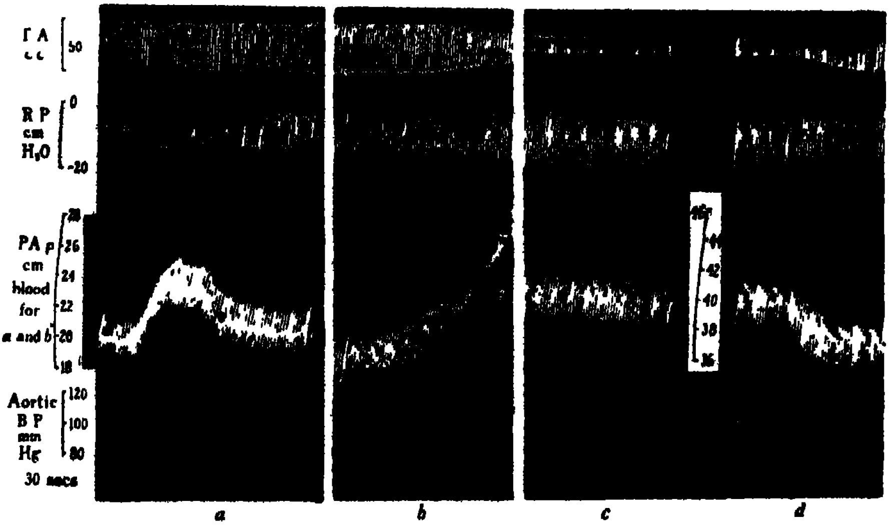

Fig 5.—Dog, 7 5 kg Lung-œsophagus preparation Simultaneous perfusion of aorta and pulmonary artery *a* Stimulation of T V S, coil distance = 7 and 5 cm *b* Ergotamine tartrate 3 0 mg injected into the pulmonary artery The P.A p. rose to 46 cm. of blood before finally falling to 40 cm *c* Stimulation of T V.S, coil distance = 3 cm *d* Adrenaline 0 15 mg injected into the pulmonary artery

(iv) *Thoracic Vagosympathetic Nerves*—Out of 13 preparations examined, stimulation of these nerves caused pulmonary vasoconstriction in 7, an effect which was reversed by ergotamine, fig. 1c, 1d, slight pulmonary vasodilatation in 4, fig 6, which was enhanced by eserine and removed by atropine, fig 7, and

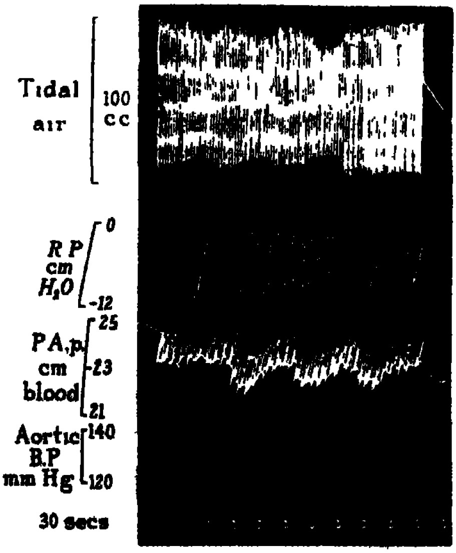

Fig. 6.—Dog, 12 0 kg. Lung-œsophagus preparation Simultaneous perfusion of the aorta and pulmonary artery Three successive stimulations of both T V.S nerves, coil distance = 8·5 cm.; subsequent to 0 3 mg. eserine.

a negative result in 2 Bronchoconstriction occurred in 9, no bronchial reaction in 2 and no tracing of the tidal air was taken in 1 experiment

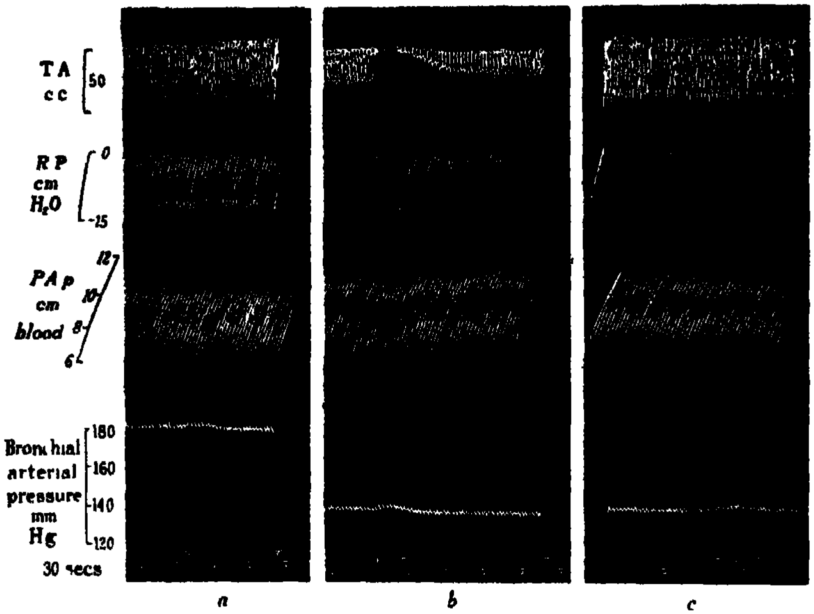

Fig 7 —Dog, 12 3 kg Perfusion of pulmonary and bronchial arteries *a* Stimulation of T V S nerves Between "*a*" and "*b*," the bronchial arterial pressure was lowered and 2 0 mg eserine added to blood reservoirs *b* 10 minutes after addition of eserine, stimulation of T V S nerves Between "*b*" and "*c*" 3 0 mg of atropine sulphate added to reservoir *c* Stimulation of T V S nerves, 9 minutes after addition of atropine Coil distance in all cases = 0 cm Amount of blood in reservoirs approximately 600 c c

The main results are summarised in the table on following page

2 *Interpretation of Nerve Stimulation Results*

Before examining the effects of the various nerve stimulations in detail, we wish to draw attention to certain conditions of experimentation peculiar to the preparations we used, which to some extent determined the sensitivity of the nerves stimulated We found repeatedly that the nerve structure furthest from the lungs lost their excitability before those situated in the region of the lung roots The excitability of the C V S nerves generally disappeared in under 1 hour, whereas we have obtained a vasomotor response on exciting the T V S nerves as long as 3 hours after the commencement of the

Table.—Effects upon the Pulmonary Vascular System and Bronchial Tubes of Stimulating various Nerve Structures

Experiment.	Pulmonary		Bronchial Tube Effects		Experimental numbers of those experiments in which no influence on the pulmonary vessels or bronchial tubes was recorded
	V C	V D	C	D	
The Cervical Vagosympathetic Nerves					
2	0	+ sl	+	0	11, 15, 16, 17 and 19 Total = 5
3	0	0	+	0	
6	+	0	+	0	
7	+	0	0	0	
8	+	0	+	0	
9	0	+ sl	+	+†	
10	+	0	0	0	
The Cervical Vagi					
9	0	+ sl	+	+†	None
10	+	0	0	0	
12	+	0	+	0	
The Stellate Ganglia					
2	+	0	0	0	8, 11, 16, 17, 18 and 19 Total = 6
6	+	0	+	+†	
7	+	0	0	0	
9	0	0	0	+	
The Thoracic Vagosympathetic Nerves					
1	0	+	+	0	15, 20 Total = 2
4	+	0	+	0	
5	+	0	+	0	
7	+	0	+	0	
8	+	0	+	0	
9	0	+	+	0	
10	+	0	0	0	
11	+	0	+	0	
12	+	0	No record		
13	0	+	+	0	
14	0	+	+	0	

V.C = vasoconstriction V D = vasodilatation C = constriction D = dilatation
† = bronchoconstriction succeeded by bronchodilatation + = positive effect. 0 = no effect
sl. = slight effect The experimental numbers in each group refer to corresponding experiments
For fuller explanation see text

perfusion. We attribute this phenomenon at any rate in part to the absence of blood supply to the remote nerve structures since the technique practised necessitated tying off the systemic vessels supplying the cervical vago-sympathetic nerves and the stellate ganglia It follows that we were unable to investigate fully the effect of excitation of the remoter nerve structures We were able to turn this loss of nerve excitability to good account, from the point of view of controlling the extent of current spread , thus stimulation of the C V S nerves immediately above the I C G , if the preparation was active, would cause a change in pulmonary arterial pressure , when the nerves had lost their excitability a further stimulation of the same intensity or greater, would produce no result whereas shifting the electrodes to the T V S nerves just below the I C G , a distance of not more than 2 cm would again cause a change in pulmonary arterial pressure, fig 8 , this means that the strength of current used even with the primary and secondary coils close together was insufficient to stimulate nerve fibres 2 cm distant Such a result is perhaps not surprising since strong currents as a rule had to be employed to alter the pulmonary arterial pressure

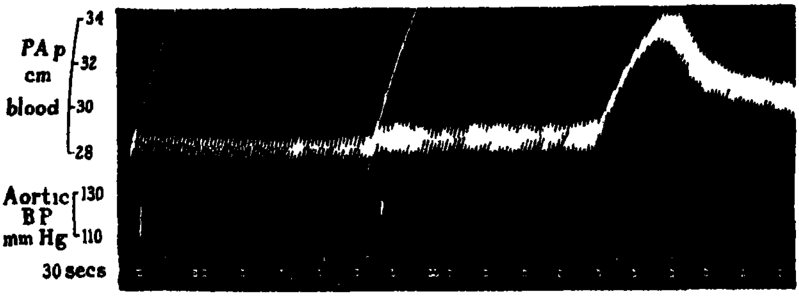

Fig 8 —Dog, 13 5 kg Lung œsophagus preparation Simultaneous perfusion of aorta and pulmonary artery Early on in the experiment excitation of C V S nerves just above the I C G raised the P A p 4 cm blood , subsequently, as shown at 1 and 2, the same nerves were stimulated (coil distance 5 and 0 cm) and gave no response. The electrodes were then shifted to the T V S nerves immediately below the I C G , which were stimulated at 3 , coil distance = 5 cm

One of the more striking observations of the experiments, is the variable response of the pulmonary arterial pressure to excitation of similar nerve structures in the different preparations , one preparation would always give rise to a vasoconstrictor response on T V S excitation and another invariably to a vasodilatator response This may be due to differences in the state of the peripheral tissues of the animals used for the preparations, to slight alterations in the technique employed, or to variations in the number of sympathetic and parasympathetic fibres in the nerve structures stimulated * In nearly all the experiments the blood from a second animal was used to fill the apparatus and the effect of the foreign blood on the peripheral nervous mechanism of the preparation is an unknown factor We have in mind the possibility that the relative sensitivities of the sympathetic and parasympathetic terminations may be determined by such factors as the foreign blood and life-history of the animal, and these may account in part for our results We do not consider the type of response to be determined by the strength or duration of the faradic stimulus because a vasoconstriction or vasodilatation once obtained did not undergo qualitative alterations with a change in the stimulus except under the influence of drugs The anæsthetic used in nearly all experiments was chloralose, 0 1 g per kilo body weight being injected intravenously after preliminary induction with chloroform and ether The possibility that the type of response is dictated by the condition of nerve ganglia situated between the point of excitation and the periphery must also be considered, and in this connection we draw attention to the work of Braeucker (1926) and also of Braeucker and Kümmel (1927), who showed in the case of the bronchomotor fibres that two successive stimulations of the same nerve separated only by a short time interval, the current strength being unaltered, may give rise to diametrically opposed effects on the bronchial calibre They suggest that the effect is not due to two different kinds of nerve fibres but to the changing response of the ganglia in the bronchial wall It is possible that intrapulmonary ganglia situated on the course of the vasomotor nerves might act in the same way, although the existence of such ganglia is at present doubtful (Larsell, 1921). But ganglia of the pulmonary plexuses or of the larger bronchi may give rise to postganglionic vasomotor fibres, for Larsell (1921) has shown that the smaller arterial branches receive twigs from the nerve plexus around the smaller bronchi which accompany them If we make the assumption that we are stimulating nerve trunks containing sympathetic and parasympathetic fibres and that the response obtained is only determined by the condition of the peripheral mechanism and by the relative number and excitability of fibres stimulated in each preparation, then the interpretation of our results is rendered less difficult We propose tentatively to make this assumption, with the proviso that the various substances injected for the purpose of testing the

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* The variable response to excitation of similar nerve structures in the preparations might be explained on the basis that simultaneous vasoconstriction and vasodilatation takes place in different parts of the vascular bed, the pulmonary arterial pressure response would then depend upon the preponderating resistance change

nature of the fibres excited and which are known to have a definite pharmacodynamic action on the peripheral mechanism, have not altered the response to nerve excitation by virtue of their action on ganglionic cells

3 *Pulmonary Vasodilatation*

The weak pulmonary vasodilatation following thoracic vagosympathetic nerve stimulation is enhanced by eserine and suppressed by atropine These reactions support the view that parasympathetic pulmonary vasodilator nerves are present in the thoracic vagosympathetic trunks (Anderson, 1905, Prevost and Saloz, 1909), and it is of more than passing interest to note that, unlike the vasodilator effects of chorda tympani stimulation and of sensory fibres stimulated antidromically (Reid Hunt, 1918, *b*), atropine suppresses the pulmonary vasodilatation Evidence has gradually been accumulating in favour of the view that the effects of parasympathetic nerve stimulation on the heart (Loewi, 1921, Loewi and Navratil, 1926), on the vascular system and on contractures of denervated muscles (Dale and Gaddum, 1930) are due to the liberation of a choline ester. Dale and Gaddum (1930) in a critical investigation and review of the evidence deal with certain difficulties raised by Gasser (1930) against accepting this view, and give expression to the opinion that in all probability acetyl choline is the ester concerned, as indeed was suggested by Dale and Dudley (1929) who obtained it from fresh animal tissues

Dale and Gaddum base their opinion to a large extent on the action of eserine which limits the choice among known substances producing both vaso-dilatation and contractures of denervated muscle to the choline esters The discrepancy between the action of atropine on the vasodilator effects due to acetyl choline and those due to nerve stimulation was met by the suggestion that atropine acts as a barrier which a choline ester artificially applied cannot pass, whereas if such an ester is liberated at the parasympathetic nerve endings, the latter will be paralysed completely by atropine only if the liberation takes place wholly without the barrier, no paralysis taking place if the liberation is within the barrier

Since we find that nerve stimulation and acetyl choline, fig 9, weakly dilate the pulmonary vessels, the effect in each case being slightly enhanced by eserine and suppressed by atropine, the probability that we are dealing with a phenomenon due to liberated acetyl choline appears to be as great as in the case of the contracture response of denervated muscle. It is true that the amount of acetyl choline necessary to produce a weak pulmonary vasodilatation is large, 1 0 mg in the non-eserinised preparation being ineffective in some experiments, but this is probably due to its destruction in the blood (Galehr and Plattner, 1927). Generally, we have observed a non-effective dose of acetyl choline becomes effective after eserine but even in the eserinised preparation approximately 0·1 mg. is the minimum dose which will produce a vasodilator effect. It should be mentioned that although vasodilatation due to acetyl choline occurred in six experiments, we encountered in one preparation, in which the pulmonary artery alone was perfused, a vasoconstriction which was enhanced by eserine and suppressed by atropine. This suggests the presence of vasoconstrictor fibres parasympathetic in nature, but since the preparation was inactive to nerve stimulation no further information was able to be obtained. Hunt (1918, *a*) found that large doses of acetyl choline added to Ringer solution perfusing the lungs (cat, rabbit, guinea-pig) reduced the outflow, an effect not prevented by atropine.

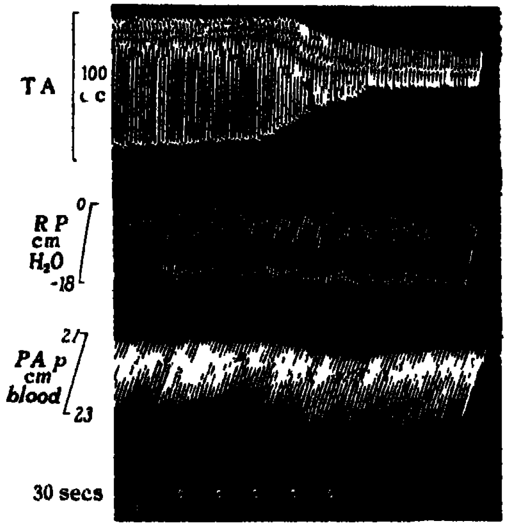

FIG. 9.—Dog, 8·0 kg. Perfusion of the pulmonary artery. Acetyl choline hydrobromide, 0·4 mg. into pulmonary arterial tubing had no effect. Eserine, 0·6 mg., was then injected and the tracing shows the effect of a subsequent dose of 0·1 mg. acetyl choline hydrobromide.

The vasodilator effects following excitation of the cervical vagi and cervical vagosympathetic nerves are not very marked and do not last long enough in the preparations for such a complete pharmacological examination as that carried out on the thoracic vagosympathetic nerves, but that the cervical vagosympathetic nerves carry parasympathetic pulmonary vasodilator nerves is indicated by the action of atropine which suppresses their activity, fig. 2

4 *Pulmonary Vasoconstriction*

In the case of the vasoconstriction response caused by excitation of the thoracic vagosympathetic nerves we have been able to perform a fairly complete examination The suppression or reversal of the effect by ergotamine (Dale, 1906, 1913) and its reproduction by the administration of adrenaline, which in turn is reduced or suppressed by ergotamine, places the fibres stimulated as being sympathetic in origin (Langley, 1901 , Elliott, 1904 , Dale, 1913) With regard to the action of adrenaline we find that vasoconstriction in the concentrations injected is the usual effect The work on the effect of adrenaline on the pulmonary vessels has been reviewed by Schafer (1921) and Wiggers (1921) The crystalline product (B W and Co Tabloid) has been used in doses varying from 7 to 150 γ in sixteen experiments , in all except one, which was negative, pulmonary vasoconstriction, indicated by a rise in pulmonary arterial pressures, occurred, and in those experiments in which it was tried, ergotamine suppressed or reversed the effect, fig 10 , in this respect we confirm Tribe (*loc cit*) The injections were made consecutively into the pulmonary and bronchial circulations

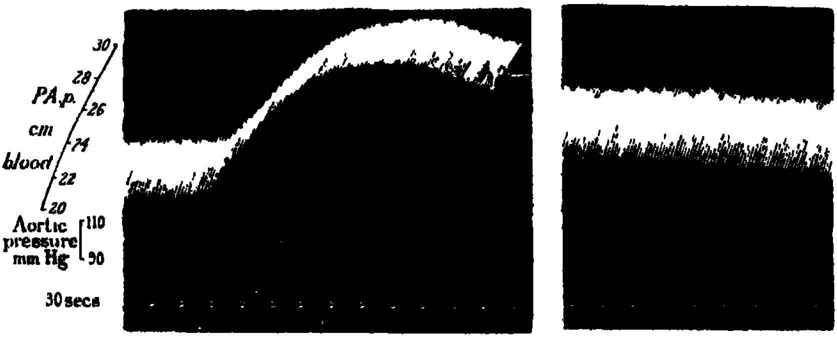

Fig 10—Dog, 7 0 kg Lung œsophagus preparation Simultaneous perfusion of aorta and pulmonary artery *a* Adrenaline 0 05 mg. injected at signal into the pulmonary artery Between "*a*" and "*b*" 3 0 mg ergotamine tartrate injected into the pulmonary artery *b* Adrenaline 0 3 mg injected into the pulmonary artery The tidal air underwent no change

Concerning the nature of the fibres running in the cervical vagi or cervical vagosympathetic, stimulation of which causes pulmonary vasoconstriction, we have no information Their excitability did not last long enough for an extensive examination to be made , the same is true for the stellate ganglia excitation experiments

5 *Bronchomotor Effects*

The prevailing effect of cervical vagosympathetic, vagal and thoracic vagosympathetic excitation is bronchoconstriction, in a few experiments a weak dilator response has followed the initial constriction Eserine enhances and atropine removes the constrictor response whether it be due to nerve stimulation or acetyl choline, figs 7, 9 The literature relating to bronchoconstrictor fibres of parasympathetic origin has been recently reviewed by Macklin (1929) and it is sufficient to state that our results are in agreement with a large number of other workers

IV. BRONCHIAL VASOMOTOR NERVES

As far as we are aware, the activity of vasomotor nerves to the bronchial vascular system has not been demonstrated, although the nerves accompanying the bronchial arteries have been described (Reisseisen, 1822, Kolliker, 1852, Berkley, 1893, Larsell, 1922, Hovelacque, 1927, Berry, Brailsford and Daly, 1931) According to Hovelacque (*loc cit*) the sympathetic twigs in man arise usually from the sympathetic ganglia D3, D4, and accompany the bronchial arteries, nerve endings do not appear to have been demonstrated in these vessels in the dog An examination of the tracings already presented to show the changes in pulmonary arterial pressure caused by nerve stimulation, reveals, in a large number of experiments, concomitant variations in the aortic pressure Nearly all the experiments are carried out on lung-œsophagus preparations in which the aortic blood supplies the tracheal, the bronchial and the œsophageal vascular beds The rise in aortic pressure during stimulation of the C V.S, C V, S G, and T V S nerves (figs 1 to 4) is due therefore to vasoconstriction in one or more of these three vascular territories

The rise in aortic or bronchial arterial pressure in response to nerve stimulation is small, and with the small blood flow through the systemic circulation of the preparation, gives little evidence of the degree of vasoconstriction With the Dale and Schuster pump putting out 30 c c per minute at 140 mm Hg against an artificial resistance maintained by a screw clip on a rubber tube fitted in place of the systemic resistance, complete occlusion of this tube raised the pressure to 182 mm Hg and naturally reduced the outflow to zero, the pump output at each beat merely served to distend the rubber connections between the pump and screw clip Thus, with blood flows of this order through the bronchial circulation, complete vasoconstriction would only raise the bronchial arterial pressure 42 mm Hg so that pressure rises of 10 mm Hg or more which we have recorded denote a considerable vasoconstriction

Attempts to demonstrate the participation of the bronchial vascular bed by excitation of the nerves before and after removal of the lungs were not entirely successful In two experiments in which stimulation of the T V S nerves caused a rise in aortic pressure, clamping the lung roots removed the response, thus indicating that the tracheal and œsophageal vessels took no part in the pressure rise A third experiment conducted on the same lines did not annul the pressure rise so that either the tracheal or œsophageal vessels or both were responsible, fig 3 In order to reach a definite conclusion on the point an isolated lung preparation with the bronchial arteries perfused directly was set up in the manner described by Berry and Daly (*loc cit*), two such experiments were performed Stimulation of the T V.S nerves raised the bronchial arterial pressure and this effect was not suppressed by atropine, fig 7, moreover, the rise in bronchial arterial pressure due to adrenaline injection was reduced by ergotamine, fig 11 Since we have been able to show that the aortic pressure rise in lung-œsophagus preparations due to T V S excitation is removed by ergotamine, fig 1, there seems little reason to doubt that the bronchial arteries are supplied with sympathetic vasoconstrictor fibres. The vasoconstrictor effect obtained by stimulation of the C V S, C V and S.G nerves have not been repeated on the perfused bronchial artery preparations, and until this is done it will not be possible to state whether the bronchial vascular bed takes part in the constriction

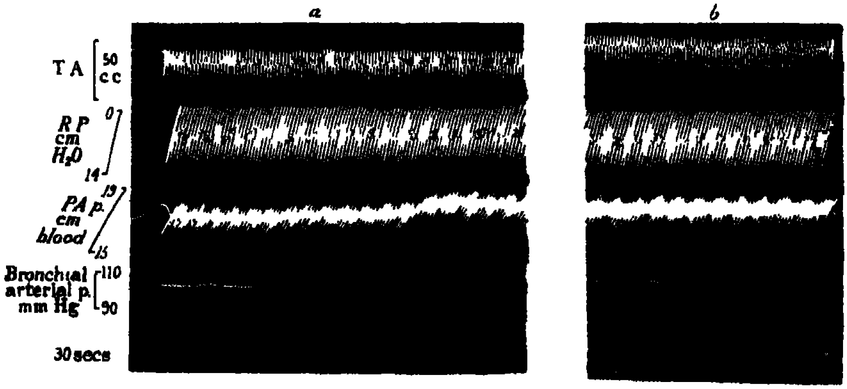

FIG 11—Dog, 9 2 kg Isolated lung preparation Simultaneous perfusion of right posterior bronchial artery and pulmonary artery. *a.* Adrenaline 0·03 mg injected into the bronchial artery (first signal) Adrenaline 0 007 mg injected into the pulmonary artery (second signal) Between "*a*" and "*b*," 3 0 mg of ergotamine tartrate was injected into the bronchial artery *b* Adrenaline 0 03 mg injected into the pulmonary artery 1 minute before commencement of tracing with no effect At first signal 0 03 mg adrenaline injected into the bronchial artery At second signal stimulation of the T V S., coil distance = 0 cm 15 minutes after ergotamine injection

V DISCUSSION

The main outcome of the investigation is the acquisition of evidence pointing to a powerful vasomotor control of the pulmonary vascular bed The mean effective pulmonary arterial pressure may increase 40 per cent, fig 1, following excitation of the thoracic vagosympathetic nerves, which on a percentage basis compares favourably with the rise in systemic arterial pressure obtained on stimulating the distal end of the splanchnic nerves Our experience of the perfusion preparation we used suggests that it is not unlikely that a much larger vasoconstriction response might be obtained with improved technique in the direction of maintaining a better blood supply to the nerves concerned, indeed, unless bronchial arterial perfusion is carried out simultaneously with the pulmonary perfusion, the nerves of the preparations are either inexcitable or only sensitive for a very short time We find that the vasodilator response is less than the vasoconstrictor, and also the enhancement by eserine of the vasodilator effect produced by nerve stimulation or by acetyl choline, although definite in the direction of prolonging the response rather than accentuating it, is not very marked It may be that the vasodilator nerves in the whole animal are less effective than the vasoconstrictor, but in the absence of further evidence we hesitate to attach significance to a quantitative distinction of this nature, since slight differences in the method employed might reveal a reverse quantitative relationship in part depending upon the initial calibre of the blood vessels. In this connection, the capillaries and perhaps the smaller arterioles and venules are more dilated under negative pressure than they are under positive pressure ventilation, and with the former type of lung ventilation a greater effect would be expected from exciting vasoconstrictor fibres than with the latter type

It has been suggested that the pulmonary vasomotor effects as evidenced by changes in the blood flow following nerve excitations are due to concomitant bronchomotor changes (Hess, 1930) and it might at first sight be argued that the pulmonary blood pressure variations we have described are due to the same cause An examination of the Table shows that the pulmonary pressure changes are independent of those taking place in the bronchial tube calibre, thus the pulmonary arterial pressure may rise, fall or undergo no change during a bronchoconstriction due to nerve excitation Such events would be hard to explain on the supposition that the bronchomotor events produced the blood pressure alterations by mechanical influences alone

VI Summary

(1) Excitation of the vasomotor nerves to the lungs may exert a profound effect on the pulmonary arterial pressure

(2) The vasomotor nerves to the lungs quickly lose their excitability in isolated preparations perfused through the pulmonary artery

(3) In isolated preparations, with simultaneous perfusion of the bronchial and pulmonary vascular beds, the excitability of the nerve structures receiving blood from the bronchial arteries may last as long as 3 hours after commencement of the perfusion

(4) Excitation of the thoracic vagosympathetic nerves may produce strong pulmonary vasoconstriction or weak pulmonary vasodilatation The pharmacological evidence points to the presence in these nerves of sympathetic vasoconstrictor and parasympathetic vasodilator fibres

(5) Excitation of the cervical vagi or cervical vagosympathetic nerves gives rise in some preparations to pulmonary vasoconstriction, in others to weak pulmonary vadodilatation The vasodilator effect following cervical vagosympathetic excitation is probably due to stimulation of parasympathetic fibres The vasoconstrictor response has not been investigated

(6) The thoracic vagosympathetic nerves contain sympathetic vasoconstrictor fibres to the bronchial arteries.

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The Origin of Erythrocytes in Herring (Clupea harengus).

By C C John, M A, Huxley Research Laboratory, Royal College of Science

(Communicated by E W MacBride, F R S —Received November 24, 1931.)

[Plates 1, 2]

The origin of erythrocytes in fishes presents an almost unique problem on account of the different methods of their formation and the varying stages of development at which they are found in the circulation The general opinion would seem to indicate that all blood cells arise from mesenchymal type of cells at a very early period in the embryonic development For a precise comprehension of the development of the blood and vascular system it is only necessary to consider the opinions of some of the recent workers on the subject According to Swaen and Brachet (1901) the mesoblast in the posterior half of the embryo consists of an inner primary somite portion and an outer primary lateral plate part In the mid-body region a portion of the primary lateral plate part adjacent to the somites divides off and forms a mass of intermediate cells The primary lateral plate grows towards the median line pushing its way beneath the somites to form a keel-shaped mass which cuts off the somites from the peritonial cavity This keel-shaped mass of cells unites with similar cells from the opposite side, resulting in a large median group of intermediate cells This same process takes place in the posterior region so that the intermediate cell mass becomes a continuous layer From this definite region of the mesoblast called the intermediate cell mass originates the heart, vascular system, and the blood in the embryo.

Though the mesodermal origin of the vascular system and the blood cells is generally accepted, the exact process of the formation and development of the different kinds of blood cells is a highly disputed problem Some of the earlier workers have maintained that all the different kinds of blood cells originate from the same types of mesenchymal cells According to this view, the mesenchymal cells change into stem mother cells which, on the one hand, give rise to leucocytes and on the other, to typical erythrocytes. This point could not be verified by any study of normal material, as circulation of the embryonic fluid is established very early before the appearance of blood corpuscles, so that when the latter are formed, they are swept into the circulating

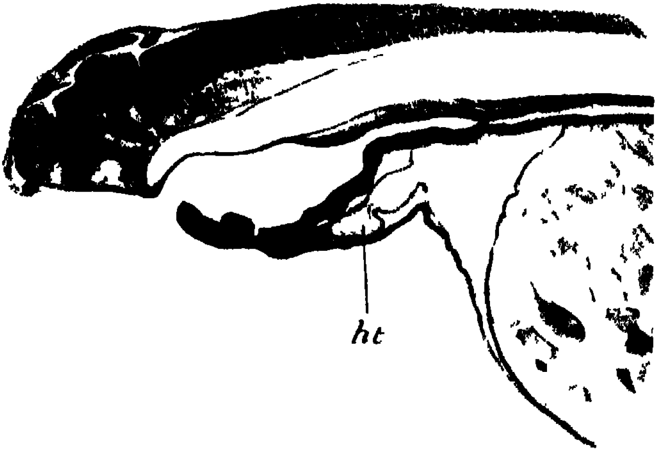

FIG. 1

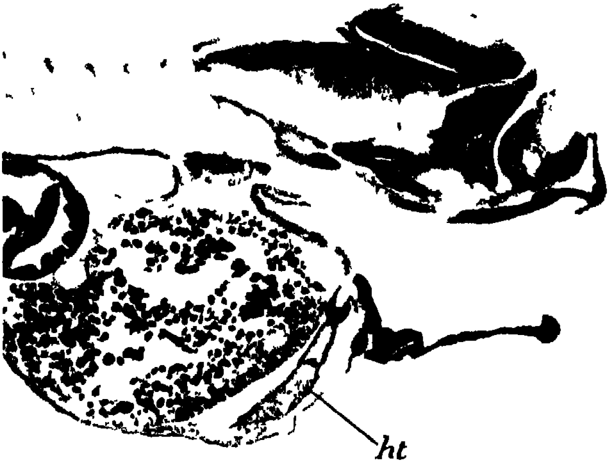

FIG 2

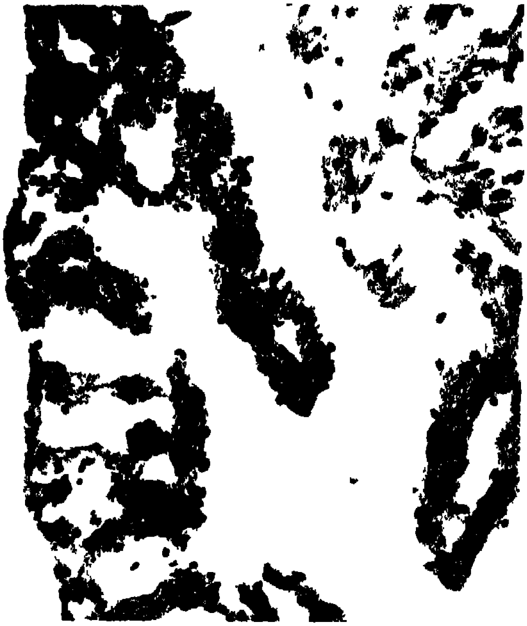

Fig 3

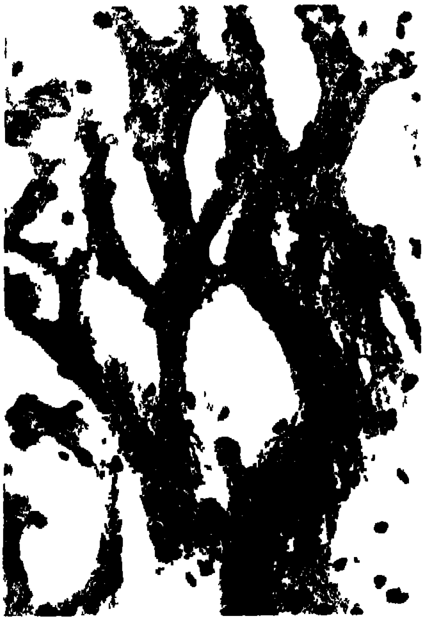

Fig 4

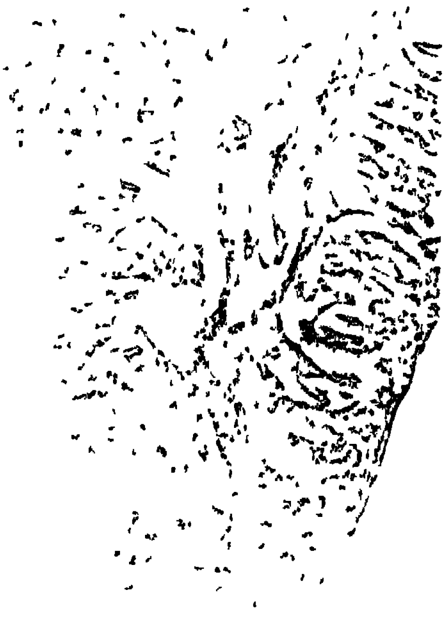

Fig 5

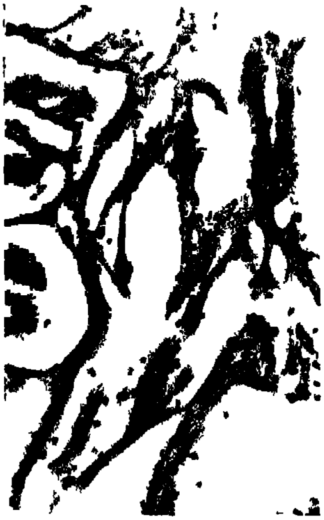

Fig 6

current and carried to all parts of the body. This necessarily makes the study of the origin of blood cells a rather difficult problem.

Stockard (1915) carried out an elaborate experimental study on the embryos of Fundulus and observed that the leucocytes and erythrocytes have different origins He treated Fundulus eggs with weak solutions of alcohol (1·5–3·5 c.c alcohol in 50 c c. of sea water) and the embryos which developed in these solutions never established a blood circulation, though in other respects they remained normal. The blood cells developed, but they were stationary in their respective places of formation and thus afforded ample scope for studying their origin It has been shown that the chief place of erythroblast-formation is the intermediate cells mass which extends from the level of the anterior portion of the kidney into the tail of the embryo and the yolk-sac blood islands, which are formed by the migration of wandering cells at a very early period. The chief source of the origin of erythroblast is therefore seen to be the intermediate cell mass which was previously described by Swaen and Brachet (1901) and Felix (1897), but contrary to their conclusion it has been shown that the mesoblast cells of the intermediate cell mass, instead of being indifferent stem mother cells which gave rise to both erythroblasts and leucocytes, definitely form only the erythroblasts

The mesenchymal cells of the intermediate cell mass are at first undifferentiated and lie between the notochord and the intestine without any endothelial envelope. As development proceeds the central mass of cells go to form the red blood corpuscles, while the cells about the periphery of this mass, which are different in nature, form the vascular endothelium.

In the adult, blood formation takes place in the hæmocytopoietic tissue represented by the spleen and bone marrow In Amphioxus a spleen is lacking and erythrocytes do not occur In the next higher type, the Cyclostomes, a hæmocytopoietic tissue occurs on the wall of the intestine which may be designated as a spleen (Jourdan and Speidel, 1930). Up to the level of the Teleosts, the spleen maintains a multiple functional capacity being hæmocytopoietically active, but in the higher vertebrates the appearance of long hollow bones is correlated with the loss of hæmocytopoietic activity of the spleen, the multiple functions of which, are taken up by lymph nodes and bone marrow In the higher forms the spleen exercises a hæmocytopoietic function only in the late embryonic and foetal stages, while in the adult it serves only as a place for the origin of lymphocytes and as a blood reservoir.

The following account deals with the origin of erythrocytes in Herring. It has been observed for a long time that the larval Herring are transparent and recall the condition of the Leptocephalus, but the cause of this transparency remained uninvestigated until now Professor E W MacBride, F R S, seems to be the only person who suspected that the transparent condition is correlated with the lack of red blood and this work was undertaken at his kind suggestion

The Herring occupies a unique position amongst the Teleostean fishes in possessing certain primitive characters during its development In the first place it stands out conspicuously by the fact that the upper jaw develops very late (Wells, 1922) and secondly by the fact that the larval stages are devoid of red blood, which appears only some months after hatching These distinct stages in development are marked by certain differences in structure and habits, so that in order to understand the relation between habits and structure, and the stage at which red blood appears, it is necessary to give a short résumé of the life-history of Herring

The egg of Herring has a diameter of 0 92–1 mm and is surrounded by a multilaminar membrane When the eggs are extruded, the egg-membrane is covered over by a layer of viscid material which causes them to adhere together and to any foreign object They are deposited upon the sea-bottom and stick firmly to the surface of any foreign object upon which they happen to rest

After fertilisation the egg shrinks away from its membrane, which now forms a capsule filled with fluid The protoplasm now separates from the nutritive yolk and forms the blastodisc, and segmentation proceeds in the ordinary way Development is rapid and the embryo reaches three-quarters round the yolk in about 1½ days On the fourth day it shows signs of movement and on the seventh day hatches as a tiny larva which is about 5 2–10 mm long It has an attenuated form, with a conspicuous yolk-sac near the anterior end The body is colourless, and transparent, the mouth gapes widely and the eyes are deeply pigmented The yolk-sac is the only source of nutritive material which is absorbed through the vascular system Still keeping to the sea-bottom the larva develops rapidly, gaining considerable strength and activity

At about the end of 3 or 4 days the yolk-sac is completely absorbed and the larva is thrown upon its own resources This transition marks the stage of migration from deep sea water and it is accompanied by certain physiological and morphological changes The larva, which is now about 10 mm long, commences its independent existence By this time the continuous embryonic fin has disappeared leaving a permanent dorsal fin near the anterior region of the tail There is still a trace of the median fin on the ventral surface between the tail and the anus in the position where the permanent anal fin appears later The gills with their supporting arches have undergone important changes leading towards the adult condition and the upper jaw begins to develop The larvæ are usually found in mid-water till they reach a length of about 16 mm during which time they undergo very little change, except for the appearance of the characteristic pigmentation

When the larva has reached a length of about 25 mm it moves to the surface, where after a short period, it migrates towards the shore The body now becomes stouter, the anus moves further forwards, and the anal fin has appeared The pelvic fin arises about half way between the anus and the gill-slits The longitudinal row of black dots on the wall of the abdominal cavity becomes very conspicuous and finer black pigment-spots appear on the dorsal surface, especially on the roof of the brain and on the tail The body becomes progressively opaque and shortly after this a silvery lustre appears first on the operculum and later along the lateral line The young Herring is now known as the "Whitebait" The appearance of the silvery sheen on the head and lateral line marks the end of a definite stage in the life-history, for at this transition into the whitebait stage red corpuscles begin to appear in the blood

During the embryonic and larval stages the blood that circulates through the body is a colourless fluid which carries nutriment and functions like the blood of Amphioxus In artificially fertilised embryos the heart has been observed to be pulsating from a very early stage, but it has never been noticed by any previous worker that the fluid which circulates in the vascular system is colourless and devoid of red corpuscles

A similar condition occurs during the development of Eel The Leptocephalus larva of the Eel is of a glassy transparence and from sections which were kindly lent to me by Mr J R Norman I have been able to find out that it is devoid of red blood In the common Eel *Anguilla vulgaris*, the larva reaches a maximum length of about 78 mm before it begins to metamorphose Up to this stage erythrocytes are completely absent. During metamorphosis the larva changes considerably in shape and becomes opaque Transverse sections of the heart at this stage show a few isolated cells in the blood These cells are circular in outline with staining nuclei and probably represent erythroblasts Typical red corpuscles are seen abundantly in the "elver" stage It has not been possible to determine the exact stage at which corpuscles appear It would have afforded a very interesting comparative study to find out the place of origin of the corpuscles, but Mr Norman's sections were originally made to study the development of the chondrocranium and are therefore not very helpful for the purpose

In the present account the larval and metamorphosing stages of Herring are referred to by their respective lengths, but their probable age in relation to length can be estimated from the accompanying table

Length in millimetres	Age in months	Length in millimetres	Age in months
15	1	35	5
20	2	40	6
27	3	44	7
30	4	47	8

The earliest larval stage which I obtained is about 6 mm long The yolk-sac is large, showing that the larva is not more than 1 or 2 days old The heart is a simple straight tube, which is divided into an anterior and posterior chamber by a constriction in the middle and lined by two layers of cells, an outer myocardium and an inner endocardium, Plate 1, fig 1, *ht* The two layers are separated by a narrow space between them A comparison of the heart of the larval Herring with that of larval Blennies which also, like the Herring, possesses demersal eggs, shows that there are certain marked differences in the structure and position of the heart between the two In the Herring larva the heart is situated below the pharynx parallel to the long axis of the body, and opens posteriorly into a wide space between the yolk-sac and body wall, Plate 1, fig 1 The structure of the heart is very simple The posterior chamber, the atrium, communicates with the space surrounding the anterior end of the yolk-sac by a wide funnel-shaped opening The fluid in this space, which is the larval blood, is colourless, but in sections it stains very lightly with hæmatoxylin, so that vascular spaces can be easily distinguished In Blennies the heart is a long cylindrical tube which is situated almost vertically in front of the yolk-sac, Plate 1, fig. 2, *ht*, and the atrium opens in the ventral part of the yolk-sac Corpuscles have already appeared and are present in large numbers in the heart cavity

During the migration of the larval Herring through mid-water to the coast the heart assumes its normal shape At the 25 mm stage the heart is a conical organ in which the myocardium is very thin, but the endocardium has developed into a syncytial sponge-work which fills the greater part of the cavity. In this syncytium there are numerous nuclei, which are large and spherical This structure of the heart prevails till the metamorphosing stage is reached. The length of the young Herring at the metamorphosing stage shows two distinct types In one type the change commences when the larval Herring is 33 mm long, and in the other, when it is 35 mm long

The metamorphosing stage marks the commencement of the change in the composition of the blood As the changes which take place are the same, in both the 33 mm and 35 mm types, we shall describe only one of the types In the 35 mm stage the larval Herring is at first rather opaque and the blood is colourless The body becomes stouter and as the first signs of the silvery sheen appears on the operculum a few erythroblasts are seen in the cavity of the heart Before any further increase in length is noticed, the young Herring is completely metamorphosed and the vascular system is crowded with erythrocytes During the metamorphosing period three distinct stages could be noticed The first opaque stage, in which there are only very few erythroblasts in the vascular fluid, the second transitional stage, when the silvery sheen begins to appear and the vascular fluid contains large numbers of erythroblasts, and the third, the silvery stage when the vascular system contains typical erythrocytes

The origin of red corpuscles in Herring is a very interesting problem, but, in studying it, one is confronted with the lack of adequate specific methods for identifying hæmocytopoietic tissue in the larval and post-larval forms During the early stages of development, most of the tissues are characterised by the presence of large nuclei which are alike, so that it is difficult to say which of them is really hæmocytopoietic In Cyclostomes the spleen is the hæmocytopoietic organ and Jourdan and Speidel (1930) maintain that it is generally so in all animals without long-bones On the other hand, Schmidt (1892) observed that the blood cells (erythroblasts) originate from the vascular endothelial cells, and the results in this paper support his view In the young Herring the spleen appears on the wall of the stomach only at the 45 mm stage, but erythrocytes are present in the blood from the period of metamorphosis In preserved material it is difficult to decide whether a tissue contains blood-forming rudiment, because hæmoglobin is not present in the cells at this stage, and so the only method available is to find out whether the cells of the tissue migrate into the vascular space Large spherical nuclei appear in the endocardial spongework at about the 28 mm stage * During the successive stages

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* The identity of the large spherical cells in the endocardial spongework, with the future erythrocytes can be demonstrated by staining sections with Okajema's elective staining for erythrocytes, counterstained with Ehrlich's hæmatoxylin In sections which are prepared by this process the large spherical nuclei are of a bright golden brown colour, while the surrounding tissue of the spongework stains blue Sections of later stages in which the red corpuscles are free in the cavity, show the same characteristic staining, the corpuscles are stained brown while the endocardial tissue stains blue This method may therefore be regarded as a positive proof of the hæmocytopoietic nature of the endocardial spongework.

of development the network increases in size and the nuclei multiply rapidly Before metamorphosis the fibres of the network are very thick and the nuclei are crowded inside them If the endocardial tissue is now teased out on a slide and stained it shows these spherical nuclei quite distinct from the elongated muscle nuclei They get easily separated from the tissue and spread on the slide This behaviour suggests that these cells are finally budded off from the surface of the tissue at the time of metamorphosis in the normal course. When first formed they are situated inside the fibres, but during development they move outwards and finally reach the surface of the fibre In the 30-mm stage a large number of these hæmoblasts are found attached to the surface of the reticulous tissue fibres, Plate 2, figs 3–6, from where they sever their connection and become free megaloblasts in the colourless blood Soon after these megaloblasts are liberated from the endocardial lining they multiply rapidly and become erythroblasts Blood smears of the transitional stage shows a greater number of erythroblasts than megaloblasts In the megaloblasts the protoplasmic part is very inconspicuous, but the nucleus is large and spherical On the other hand, the erythroblast nucleus is smaller and oval in shape The protoplasm surrounding the nucleus is thicker and stains very light The cell itself is disc-like and circular or oval in outline At the beginning of the silvery stage typical erythroblasts appear They are flat elliptical discs with oval nuclei In smears a few megaloblasts and erythroblasts are also seen This completes the development of the red-blood cells and by this time the young Herring is completely metamorphosed into the silvery stage

One interesting feature in the development of Herring and Leptocephalus is that, in both, red-blood cells begin to appear after the larvæ have reached shore, but the causes which lead towards this and their physiological significance on the origin of blood are beyond the realm of morphology It has already been stated that the presence of colourless blood in the larval stages of Herring is a primitive feature which recalls the condition of the blood in Amphioxus When the body is transparent and slender the oxygen which gets dissolved in the colourless blood is sufficient to maintain the growth and activity of the animal, but when the body becomes stouter and opaque the amount of oxygen which gets dissolved in the vascular fluid is inadequate and thus necessitates the development of cells which are specially fitted to absorb oxygen. These cells develop in the endothelial lining of the vascular system and are budded off into the vascular fluid. In a more or less general way, this gives a picture of the origin of corpuscles in vertebrates.

In Teleosteans, as a general rule, blood corpuscles appear very early in development The blood-forming rudiment is separated from the endothelium when the intermediate cell mass begins to form the vascular system, as a means of quickening the formation of erythrocytes This rapid process masks their origin, but in Leptocephalus and Herring, owing to their primitive nature, the blood cells develop very slowly and thus give the clue to the origin of erythrocytes from an evolutionary point of view

Though it was the aim of the author to study the physiological aspect of the origin of blood in Herring, the difficulty of keeping the larvæ alive, rendered it an almost impossible task

In conclusion, I wish to express my deepest gratitude to Professor E W MacBride, F R S , under whose guidance and supervision this work was carried out I wish to thank also Mr E Ford, Fisheries Naturalist of the Marine Biological Laboratory, Plymouth, for providing me with the necessary material of Herring, and Mr J R Norman for kindly lending me his sections of Leptocephalus

EXPLANATION OF PLATES

PLATE 1

FIG 1 —Longitudinal section of the head of Herring larva (about 2 days old), showing the position of the heart

FIG 2 —Longitudinal section of the head of Blennies larva, showing the position of the heart and the corpuscles inside the heart cavity

PLATE 2

FIGS 3 and 4 —Sections of the endocardium showing the origin of hæmoblasts and their migration to the surface of the fibres

FIG 5.—Section of the ventricle showing the structure of the endocardium

FIG 6 —Part of the same section highly magnified to show the hæmoblasts

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Intracellular Hydrion Concentration Studies. V.—Colorimetric p_H of Malignant Cells in Tissue Culture.*

By Robert Chambers, Washington Square College, New York University, New York, and R J Ludford, Laboratories of the Imperial Cancer Research Fund, London

(Communicated by J A Murray, F R S —Received December 8, 1931)

[Plate 3]

The microinjection of the relatively small cells of mammalian tissues offers considerable difficulties, the chief of which is some means of knowing whether the cell is still alive after the injection Usually deterioration of the cell is made evident by easily recognisable morphological changes, but this is not always the case The best criterion so far encountered for the injection of solutions of dyes is the restriction of the colour to the cell injected This is more difficult to judge with dyes, especially those basic in nature, which easily penetrate cells from without In such cases special pains must be taken to prevent spilling into the medium during the time that the pipette is being brought into the cell to be injected Fortunately, the Clark and Lubs indicators are sodium salts of acid dyes, and most of these penetrate living cells, either very slowly, or not at all The consequence is that a small amount accidentally spilled about the cells quickly fades away by diffusion into the surrounding medium and does not disturb the procedure of injection

When once a solution of an acid dye is injected into a cell, the acquired colour persists for an appreciable length of time Up to the present, the injection of cells of somatic tissues has always been followed, generally within 5 to 15 minutes, by observable signs of cytolysis This is accompanied, and frequently preceded, by a fading out of the colour of the injected dye. From these observations it has been assumed that the cell is alive as long as the indicator is still within the cell injected This assumption is supported by experiments on marine ova (Needham, 1926), for they have been demonstrated to be alive while retaining the colour of the injected indicator both in the cytoplasm and in the nucleus (Chambers and Pollack, 1927)

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* This communication forms No V of a series in which one of the authors (R. C.) has been concerned, of which the earlier numbers have been published elsewhere.

In all the various types of cells studied where these precautions have been taken, the colorimetric value of the cytoplasmic p_H has been found to lie within one or two decimal points of 6·8, and the p_H of the nucleus to be equally close to 7 6 (Chambers, Pollack and Hiller, 1927, Chambers, 1928, *ibid*, 1929, 1930)

In regard to the technique of microinjecting tissue cells, probably the chief difficulty lies not so much in the small size of the cells as in the minuteness of the tip of the pipette It is comparatively easy to draw in and to expel aqueous solutions by means of a pipette with an aperture of less than half a micron in diameter, but the chances of clogging are very great as the tip is being pushed through the glutinous material of the cell to be injected When once a pipette is clogged the only remedy is to break off the tip, which then is usually too large for proper service

Fig 1, Plate 3, is from a photograph to show the tip of a serviceable though relatively coarse pipette inserted into the nucleus of a mammary carcinoma cell Note that there is a pronounced taper of the pipette at its tip It is this kind of a taper which makes it possible to control, in some measure, the minute amounts of fluid to be ejected

The first cell injected was a spread-out cell of the mammary carcinoma 27,* one of a sheet of cells growing in a plasma culture The pipette, containing a standard 0 4 per cent aqueous solution of phenol red, was brought into the cytoplasm immediately beneath the nucleus The indicator slowly spread from the tip of the pipette and stopped at the cell boundary The nucleus acquired a rose red colour, while the surrounding cytoplasm was tinged a distinct yellow As far as could be seen, none of the dye escaped from the cell. The colour lasted for less than half a minute, after which it began to fade One minute later the colour disappeared Both during and after injection the nucleus showed none of the effects of injury which usually occur when it is punctured (Chambers and Fell, 1931, Chambers and Ludford (*in press*)). Cells of the Crocker sarcoma (fibroblastic) were injected with similar results

Phenol red was injected also into normal mammary gland cells These cells were obtained from tissue cultures of the mouse mammary gland and the injections were made in order to compare a normal cell with the carcinoma 27 cell In the several cases in which the injections were successful the nucleus always acquired a red colour and the cytoplasm, a faint yellow

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* The nomenclature of the various types of tumour cells used is that given to the tumours grown in the Laboratories of the Imperial Cancer Research Fund The senior author takes this opportunity of thanking the Executive Committee of the Imperial Cancer Research Fund for a grant to defray expenses during the period of investigation and also for the kindness and appreciative interest of Dr. J A. Murray and the other members of the Institute.

The other indicator injected was brom cresol purple, also in a concentration of the standard 0 4 per cent aqueous solution A micropipette filled with this indicator was brought into the cytoplasm of a well spread-out carcinoma 27 cell, one of a narrow tongue-like outgrowth of cells Upon injection the dye spread throughout the cell A relatively small area of the cytoplasm immediately surrounding the pipette took on a pale yellow tint, while the rest of the cell became pale blue The nucleus also became blue which gradually deepened to a distinct purple hue A photograph of this with another cell is shown in fig. 2, Plate 3 The injected cell has a darkened nucleus due to the colour of the injected indicator The nucleus of the other cell is of about the same size as that of the coloured cell but is partly masked by overlying cytoplasmic granules. It is significant that this cell, although contiguous to the injected cell, remained colourless The colour in the injected cell persisted for a minute, then the nucleus faded as it degenerated into a mottled, shrunken body, and the cytoplasm changed from blue to yellow A few minutes later the colour disappeared from the entire cell.

Cells of the tar carcinoma 2146 were injected with brom cresol purple with similar results In one case a clogged pipette was inserted into one nucleus of a binucleate cell Application of pressure resulted in a sudden outflow from the pipette, bursting the nucleus and tearing the cytoplasm The injured part of the cytoplasm acquired a vivid yellow colour, while the distant nucleus quickly coloured blue and then purple, and the cytoplasm around it, a faint blue The colour faded out within a minute and the nucleus became shrivelled.

The question as to whether it is possible to draw conclusion from the colour of p_H indicators in such a heterogeneous system as the protoplasm of cells, is discussed elsewhere (Chambers and Pollack, 1927, Chambers, 1928; *ibid.*, 1929, 1930) The chief argument in favour of doing so is the fact that the injection of all the Clark and Lubs indicators, whose colour changes are within the range of the intraprotoplasmic p_H, give consistent results irrespective of the chemical constitution of the indicator used It should also be realised that no attempt is made to narrow the limits of the p_H values beyond those determined by pronounced colour changes of the various indicators which overlap one another along the ranges of successive p_H values.

It is to be noted that the cytoplasm of the cells studied exhibited distinct signs of an acid of injury when the cytoplasm was injured This agrees with results previously described concerning the acid of injury

Another point, which has not been sufficiently discussed in previous papers, relates to the colouration of the nucleus when an acid dye is injected into the cytoplasm Basic dyes, such as neutral red, which colour cells by immersion, do so probably by virtue of the ease with which they pass from a region of greater alkalinity, *e g*, the medium, to a region of lesser alkalinity, *e.g*, the interior of the cell. Acid dyes, in general, pass in the opposite direction and it is possibly for this reason that most of them do not stain cells by simple immersion Consequently, the fact that brom cresol purple, an acid dye, readily passes from the cytoplasm into the nucleus, is an added argument for the greater alkalinity of the nucleus as compared with that of the cytoplasm

The results of our experiments, shown in the accompanying table, indicate that the cells of the mouse tumours in tissue culture, viz, mammary carcinoma 27, tar carcinoma 2146, Crocker sarcoma, and the cells in tissue culture of the normal mouse mammary gland, have similar values for their cytoplasmic and nuclear p_H, judged by the injection of brom cresol purple, and of phenol red.

Table representing colours obtained from injection of indicators into various cells Colour reactions obtained also with Crocker sarcoma are marked thus *, and with tar carcinoma 2146, thus †

Cells in tissue culture			Colours assumed on injection of indicators	
			Phenol red	Brom cresol purple.
Normal mammary gland of mouse and mammary carcinoma 27	Nucleus		Red*	Blue and purple†
	Cytoplasm	Normal	Yellow*	Blue†
		Injured	Yellow	Yellow†

In previous communications, one of us (R C) has always referred to the colour of brom cresol purple in its alkaline range as blue, instead of the purple which is its well recognised tint in a test-tube The reason for this is that the living cell tolerates only a minimal amount of the indicator, just sufficient to produce a tinge of colour. Under the microscope this appears blue. When a greater quantity is injected into the cytoplasm cytolysis sets in and the colour at once changes to a distinct yellow characteristic for the acid range of the indicator. On the other hand, the living nucleus does not change its alkaline reaction on death (Chambers and Pollack, 1927) It may receive an overdose without causing a shift from the alkaline range of the indicator, and hence the nucleus is able to receive a sufficient quantity of the indicator to bring out the purple colour.

The present investigation definitely places the colorimetric p_H of the cytoplasm of the various tumour cells between the alkaline range of brom cresol purple and the acid range of phenol red In accordance with the colour chart of Clark and Lubs (Clark, 1925) the cytoplasmic p_H lies between 6·4 and 7·0 which is in agreement with those previously found for normal cells, viz., 6 8 ± 0 1

The cytoplasm, when injured and undergoing cytolysis, is definitely yellow with brom cresol purple This indicates that the p_H of the acidity produced on injury is 5 6 or below, and approximates the value of 5·4 which has been found previously in injured normal cells

The intranuclear p_H is well within the alkaline range of phenol red, viz, definitely above 7·2. It is unfortunate that time did not permit further experimentation to observe the effects of injecting cresol red This indicator would give the alkaline limit for the p_H of the nucleus. In the injured nucleus brom cresol purple keeps its blue or purple colour, and phenol red its red colour Hence there is no evidence for an acid of injury such as develops in the cytoplasm In this respect also the tumour cells examined are in accordance with normal cells

PLATE 3

FIG 1 —Photograph taken with 2 mm. oil-immersion objective showing tip of micropipette "*p*" inserted into hyaline nuclear area of a mammary carcinoma 27 cell in tissue culture.

FIG 2 —Two mammary carcinoma 27 cells, part of tongue-like outgrowth in tissue culture. Brom cresol purple had just been injected into the cytoplasm The deep coloured purple of the nucleus "*n*" shows dark in the photograph. The cytoplasm was pale blue. The neighbouring cell with its nucleus partly masked by overlying cytoplasmic granules, remained colourless.

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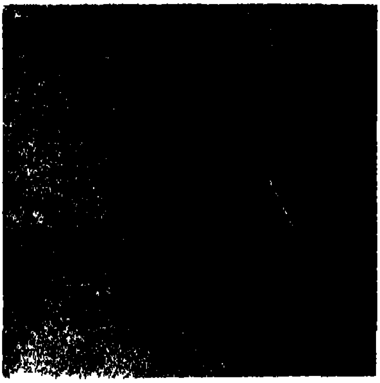

Fig 1

Fig 2

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Studies in the Nervous Control of Carbohydrate Metabolism.

I.—*The Position of the Centre*

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[Plate 4.]

The influence of the nervous system over the metabolism of the carbohydrates was demonstrated by Claude Bernard's famous piqûre experiment (1855) many years before that of hormones, but recently attention has been paid mainly to the latter. As our knowledge of the influence of hormones in general has expanded, the possibility that the activities of the glands which produce them are regulated through the nervous system has been considered, and the question arises whether it is indirectly through this control that the nervous system affects carbohydrate metabolism. It was with the object of solving this question that the present research was primarily undertaken.*

Our original plan was to investigate the nature of the changes in carbohydrate metabolism brought about by piqûre, but we very soon found that this operation, as usually practised, is uncertain in its results and, moreover, that it is impossible to differentiate these results from the disturbances in carbohydrate metabolism which anæsthesia in itself induces. This led us to adopt the method of decerebration rather than piqûre and, by its use, we have succeeded both in localising within fairly narrow limits, the part of the brain which is principally concerned with the nervous control of carbohydrate metabolism and in throwing some light on the nature of the biochemical changes over which this control is exercised. In the present paper, we will first of all give a brief account of the experiments which led us to adopt the decerebration method rather than that of piqûre, and then consider more in detail the results which have enabled us to localise the diabetic centre. In subsequent papers we will deal with the metabolic disturbances resulting from the decerebration and with the modifications of these disturbances which occur when the nervous pathways through which the centre exercises its control are interfered with.

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* We are greatly indebted to the Medical Research Council for a grant in aid of this investigation.

Experiments under Luminal and Amytal Anæsthesia.

The first experiments were performed on rabbits anæsthetised by injecting sodium luminal intraperitoneally or subcutaneously. About 0·2 gm. per kilo. in 10 per cent. solution, the amount varying somewhat in different animals, induced satisfactory and steady anæsthesia, but the behaviour of the blood sugar concentration was uncertain. In a series of five experiments on recently-fed rabbits, it rose slowly so as to reach about 0·200 per cent. in three of them and 0·460 per cent. in another. The muscle glycogen was determined in three of the animals and was found to increase in two and diminish in one.

Notwithstanding the rather unsatisfactory nature of these results, particularly with regard to the behaviour of the blood sugar percentage, it was decided to see whether piqûre of the floor of the fourth ventricle would cause hyperglycæmia in recently-fed rabbits under similar conditions of anæsthesia. The details of a typical experiment are as follows:

Experiment 8.

A rabbit (2400 gm.) was anæsthetised at 8.45 a.m. with 4 c.c. of 10 per cent. sodium luminal subcutaneously. At 10.30 tracheal and carotid cannulæ were inserted and the gracilis and tibialis anticus muscles dissected from the left leg. The floor of the fourth ventricle, exposed by opening the occipito atlantoid membrane was then punctured bilaterally with a dissecting needle, one puncture being 4 mm. and the other 5·5 mm. from the tip of the calamus scriptorius and both of them within 2·5 mm. of the mid line. Piqûre was performed at 11.15 and blood samples removed from the carotid at 11.20 and each succeeding hour until 4.20 when the blood pressure was recorded. The tibialis anticus and gracilis muscles of the right leg and a portion of liver were then removed for analysis and the animal killed by bleeding. The blood sugar percentages for each succeeding hour were as follows: 0·186, 0·162, 0·121, 0·126, 0·130, 0·126.

The arterial blood pressure equalled	70 mm. Hg
The glycogen in muscles of the left leg	0·35 per cent.
and in those of the right leg	0·30 ,,
The glycogen in liver was	0·94 ,,

Piqûre of the floor of the fourth ventricle was similarly performed on four other recently-fed rabbits anæsthetised with either luminal or amytal given intraperitoneally. Anæsthesia was not as satisfactorily maintained with amytal as with luminal, so that its injection had to be repeated in three or four hours in the experiments in which it was used. In all cases the blood sugars were determined hourly and they either fell steadily or remained unchanged during the 4 or 5 hours of the observation. The arterial blood pressure was never below 70 mm. Hg.

The absence of hyperglycæmia in these experiments stands out in sharp contrast to its almost invariable development when piqûre is performed exactly according to Claude Bernard's instructions—piqûre through the skull in unanæsthetised animals * Since involvement of the cerebellum might be responsible for this hyperglycæmia, several experiments were performed in which Bernard's method of puncturing through the skull was used in rabbits under amytal or luminal, but with uniformly negative results There can, therefore, be no doubt that amytal and luminal entirely inhibit the hyperglycæmic effects of piqûre as ordinarily performed on rabbits

These results raised the question whether other forms of experimental hyperglycæmia would fail to develop on animals anæsthetised with amytal or luminal Adrenaline and asphyxia were tried with the following results —

Table 1

No of experiment	Anæsthetic	Experimental method	Per cent blood sugar at 30 minute intervals after
16	Luminal 0 2 gm amytal 0 1 gm + 0 15 gm later	Inject 0 5 c c 1/1000 adrenalin chloride	0 268, 0 323, 0 385, +0 385
18A	Luminal 0 2 gm amytal 0 1 gm + 0 2 gm later	Mechanical asphyxia for 20 minutes	0 177 (5 minutes), 0 139, 0 106 0 088, 0 125
	Then later— B amytal 0 05 gm	0 5 c c adrenaline	0 172, 0 259, 0 325, 0 355
34	Luminal 0 2 gm, amytal 0 1 gm Luminal 0 2 gm	Ether, small amount	0 135 0 105, 0 081

The animals were kept as uniformly anæsthetised as possible, and the results clearly show that adrenaline still caused hyperglycæmia, whereas asphyxia and ether failed to do so No attempt has been made to ascertain whether the minimal dose of adrenaline necessary to induce hyperglycæmia is the same in anæsthetised as in unanæsthetised animals, and until this is known it is impossible to say whether amounts of adrenaline comparable with those which are believed to be secreted into the blood from the adrenal glands under certain conditions would be effective in animals under amytal or luminal Incidentally, the results indicate the principles of a method by which hyperglycæmia due to different causes may be differentiated We will return later to a consideration of this aspect of the problem

It was then decided to ascertain whether there is any region of the brain other than the floor of the fourth ventricle in which either puncture or more

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* This method has been employed under local anæsthesia by one of the present authors (J J R.M.) on several previous occasions (1905, 1923) and has always been found to result in very marked glycosuria or hyperglycæmia within a hour or so

extensive injury, such as transection, will cause hyperglycæmia in recently-fed rabbits anæsthetised by repeated intraperitoneal injection with luminal and amytal ‡ The degree of anæsthesia was kept as uniform as possible in the different experiments

The results are condensed in Table II in which the blood sugar percentages are given at approximately hourly intervals after making the lesion The carotid arteries were ligated, unless otherwise stated

Table II —Effect on Blood Sugar of Lesions in various Regions of Brain (Luminal Amytal Anæsthesia).

No of experiment	Nature and approximate position of lesions in fore brain	Blood sugar percentages at hourly intervals after lesion *
21	Puncture of right frontal lobe, 4 mm from mid line and 15 mm from anterior end (carotids not ligated)	*0 101* 0 127, 0 125, 0 190, 0 106, 0 115
25	Puncture in mid line extending to just behind the pituitary	*0 111*, 0 230, 0 225, 0 224, 0 230, 0 230, 0 243
26	Puncture 3 mm to right of mid line and extending to behind the pituitary	*0 104*, 0 155, 0 157, 0 178, 0 176, 0 128, 0 122
27	Puncture in mid line and extending to behind the pituitary	*0 084*, 0 094, 0 076 (½ hour), 0 073, 0 087, 0 084
28	Exposure of pituitary from side and destruction of infundibulum	*0 090*, 0 173,† 0 210 (½ hour), 0 177, 0 181, 0 140, 0 124, 0 116
30	Exposure of pituitary from side and destruction of region just behind chiasma (much basal hæmorrhage)	*0 118*, *0 218*,† 0 197, 0 175, 0 147, *0 158*, *0 114*
31	Destruction of olfactory bulb A, followed by destruction of pituitary B	*0 126* A, 0 227 (½ hour), 0 220 *B*, 0 239 (½ hour), 0 276, 0 257, 0 196, 0 145
32	Elevation of frontal lobes, *A*, then destruction of olfactory bulb *B*, and finally, decerebration involving region of pituitary *C*	*0 100* *A*, 0 172, 0 211, 0 247 *B*, 0 240, 0 230 *C*, 0 314 (½ hour, 0 320
36	Cutting olfactory bulbs and puncturing from behind chiasma to top of brain, *A*, finally decerebration, *B*	*0 105*, *0 124* *A*, 0 117 (½ hour), 0 124, 0 114 (2 hours), 0 087 *B*, 0 089 (½ hour), 0 086, 0 091
37	Cutting olfactory bulbs, *A*, puncturing from behind chiasma to top of brain, *B*, finally decerebration C	*0 102* *A*, 0 117 (½ hour), 0 140 *B*, 0 130, 0 130 *C*, 0 123, 0·124
40	Luminal, trephining at *A*, puncture involving pons at *B*	*0 100* *A*, 0 244 (½ hour), 0 283 *B*, 0 249
41	Luminal, trephining at *A*, puncture involving pons at *B*, followed by a second puncture at *C*	*0 123*, *0 157* (¾ hour), *A*, 0 215 (½ hour), 0 193, 0 196 *B*, 0 185, 0·139 *C*, 0 116 (1½ hours)
42	Luminal, trephining at *A*, puncture involving pons at *B*, followed by a second puncture at *C*	*0 098* *A*, 0 255, 0 301 *B*, 0·215, 0 189 *C*, 0 184 (½ hour), 0 139

* The first figure in italics gives the blood sugar before making the lesion When smaller intervals were used, this is shown in brackets

† This taken 1 hour later, after exposing pituitary

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‡ These anæsthetics were used in this combination, since we found that when a sufficient single dose of sodium amytal was given intraperitoneally to induce deep anæsthesia, fatal results often followed This was avoided by replacing some of the amytal with luminal.

In experiments 25, 26 and 27 the cerebrum was punctured in a line extending to just behind the pituitary gland, the blood sugar rose decidedly in one, and slightly in another In experiments 28 and 30, the pituitary region was exposed by carefully raising the lateral lobes of the cerebrum, and the infundibulum severed, or the region of the tuber cinereum destroyed, without causing the blood sugar to rise above the level to which it had been raised by the preliminary operative procedure It was found that the region of the pituitary could be more conveniently approached from in front, by cutting the olfactory tracts and slightly raising the frontal lobes In two out of the four experiments in which this was done, the blood sugar rose decidedly (Nos 31 and 32). In one of these experiments (No 31), the pituitary was then destroyed with the result that the hyperglycæmia became somewhat more pronounced, but it subsequently declined, in another (No 32), hyperglycæmia became more marked following transverse decerebration involving the pituitary In the remaining two experiments of this series (Nos 36 and 37), a curved aneurysm needle, passed from before backwards under the frontal lobes was pushed upwards just behind the chiasma and directed so as to emerge on the surface of the cerebrum In neither case was there any change in blood sugar. In view of the definite increases in blood sugar which followed destruction of the olfactory tracts in two of the experiments, the effect of electrical stimulation of the base of the brain in the region of the optic chiasma was next studied. The percentage of blood sugar, after placing the electrodes in position, was 0·137 and it fell steadily after stimulation Himwich and Keller (1930) in similar experiments on cats under amytal, found that the blood sugar sometimes became increased The last three experiments (Nos 40 to 42) show that in rabbits under luminal anæsthesia an operation, such as trephining, may, in itself, be followed by hyperglycæmia and, on the other hand, that puncture of the pons may fail to cause it

It became evident from these results, that further progress in localising the diabetogenic centre could not be made by using animals under the influence of amytal or luminal It was therefore decided to change the plan of attack on the problem and the method adopted was to perform decerebration at different levels of the brain stem in animals deeply anæsthetised by the *intravenous* injection of amytal and to observe the behavior of the blood sugar in the *decerebrated* preparation after the effects of the drug had disappeared (2 to 3 hours). That decerebration, as ordinarily performed, is usually followed by hyperglycæmia has been known for several years, and, although it is plain that, by such a method, exploration for the position of the diabetogenic centre must be restricted to the basal regions of the brain, the perfectly definite results which we have obtained are, we believe, of significance.

The procedure was to inject into an ear vein sufficient amytal,* dissolved in 2 per cent Na_2CO_3, to induce complete anaesthesia The necessary dose varied somewhat with different rabbits, the average being 55 mg per kilogram body weight The anaesthesia, which develops without any excitement or muscular activity, was usually satisfactory in 5 minutes, but if this average dose proved inadequate, a second injection of 20–30 mg per kilogram was given A tracheal cannula was then inserted, one or two small trephine holes made in the skull and a large area of the cranial vault removed by means of bone forceps By extensively exposing the brain in this manner, its compression from haemorrhage was avoided so that convulsive movements of the animal were practically eliminated Decerebration was performed with an almost straight aneurysm needle which was passed into the brain between the posterior extremity of the cerebrum and the tentorium and directed downwards until stopped by the basilar process of the occipital bone, its slope backwards varying according to the exact level at which it was desired to make the decerebration In many of the experiments, just before performing the decerebration, a piece of liver was removed and the right leg was amputated for determination of glycogen. The sugar, lactic acid, etc of the blood were then determined at about hourly intervals for a period of 3 to 7 hours At the end, amytal was again given intravenously and, as a rule, a second portion of liver was removed and the left leg amputated for determination of glycogen, after which the animal was killed by bleeding The head was then cut off and put in formalin solution In a few days it was cut longitudinally in the mid-line and the exact position of the decerebration marked on a photograph

We will confine our attention for the present to the results which have a bearing on the relationship between the level of the decerebration and the behaviour of the blood sugar They have been collected into four groups according to this level, as indicated in fig 1 Out of the 10 experiments in which decerebration was in region 1 (Table III), no significant rise occurred in blood sugar in eight, and the rise was marked in only one of the remaining experiments (No 135) In the five experiments in which decerebration was in region II (Table IV), the blood sugar percentage did not undergo any change except in one experiment (No 65) and in that only after several hours Even when the cut was in the region closely adjacent to the pons there was no hyperglycæmia. We reproduce in Plate 4 the photographs of the brains in which the decerebrations were in positions I and II.

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* We are greatly indebted to the Eli Lilly Company of Indianapolis, U S.A , for supplying us with the amytal.

The almost invariably negative character of these results on the rabbit was unexpected in view of the hyperglycæmic effects which other observers have recorded after lesions at corresponding levels of the brain in the cat or dog, and it will be appropriate here to refer to these observations. Beattie, Brow and Long (1930) state that section of the hypothalamus in cats, performed through the pituitary fossa, is followed within 24 hours of the operation by glycosuria and hyperglycæmia. This region is described as being delimited posteriorly by a line passing from the anterior edge of the superior colliculus to the posterior edge of the mammillary bodies. In our experiments in region I, decerebration involved this area in 3 (Nos. 63, 74, and 133), and it was somewhat further forward but still behind the chiasma in 5 (Nos. 135, 136, 137, 138 and 140). All decerebrations in region II were probably a little behind the hypothalamus (Nos. 59, 61, 64, 65, 85, 132). Bulatao and Cannon (1925) also found that marked hyperglycæmia developed in cats after decerebration or decortication had been performed under ether anæsthesia provided

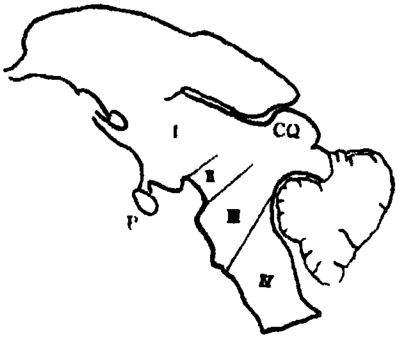

Fig. 1.—Outline drawing of sagittal section of rabbit's brain, to show regions at which decerebrations were performed.

Table III.—Behaviour of Blood Sugar following Decerebration in Position 1

No. of experiment	Normal B S	B S immediately after decerebration	B S at hourly intervals after decerebration 1	2	3	4.	5	6.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent.	per cent.
62	0 093	0 081	0 094	0 093	0 091	0 098	0 091	0 099
63	0 075	0 101	0 100	—	—	—	—	—
74	0 104	0 119	0 113	0 105	0 098	0 106	0 090	—
133	0 112	0 196	0 196	—	—	—	—	—
135	0 108	0 153	0 266	0 310	0 300	0 304		—
136	0·104	0 149	0 144	0 142	0 142	0 164		—
137	0 115	0 133	0 133	0 133	0 150	0 152	—	—
138	0 105	0 099	0 099	0 090	0 087	—	—	—
139	0 116	0 151	0 151	—	0 128	0 128	—	—
140	0 085	0 088	0 096	—	—	—	—	—
Average	0 102	0 127	0 139	0 145	0 155	0 174	0 090	—

Table IV —Behaviour of Blood Sugar following Decerebration in Position II

No of experiment.	Normal B.S	B S immediately after decerebration.	B S at hourly intervals after decerebration							
			1	2	3	4	5	6	7	8
	per cent.	per cent	per cent	per cent	per cent	per cent	per cent	per cent.	per cent.	per cent
59	0·087	0·105	0·085	—	0·126*	—	—	—	—	—
61	0·117	0·110	0·104	0·180	0·140	0·162	0·166	0·162	0·140	0·168
64	0·124	0·117	0·110	0·127	0·106	—	—	—	—	—
65	0·134	0·138	0·139	0·123	0·134†	0·168	0·250	0·207	0·206	—
85	0·178	0·142	0·129	0·090	0·107	—	—	—	—	—
132	0·101	0·141	0·183	0·176	0·169	0·151	—	—	—	—
Average	0·123	0·125	0·125	0·139	0·130	0·160	0·208	0·184	0·173	0·168

* Decerebration not complete

† Only 35 minutes

that a high degree of pseudo-affective activity became developed, otherwise there was little, if any, increase in the blood sugar level They consider that the hyperglycæmia is one of the effects resulting from a discharge of impulses from a sympathetic centre situated in the hypothalamus, this discharge being normally prevented by inhibitory influences from the cortex, an interpretation which is in unison with that of Mellanby (1919) who did not, however, sharply define the position of the "hyperglycæmic" centre Olmsted and Logan (1923) also found that decerebration posterior to the hypophysis did not usually result in hyperglycæmia in cats, although it always caused it when performed at the level of this gland

There is also evidence that electrical stimulation of the hypophysis causes the blood sugar to increase Thus, Keeton and Becht (1915) found in dogs that stimulation causes hyperglycæmia independently of the ether used for anæsthesia They state that stimulation anterior or posterior to the gland in dogs does not affect the blood sugar level

We may also refer to the experiments of Karplus and Kreidl (1927) and Houssay and Mollinelli (1925) who obtained evidence that electrical stimulation in the region of the hypothalamus resulted in a great increase in the internal secretion of adrenaline, which would indirectly raise the blood sugar level. The latter authors state that a similar effect can be obtained by stimulating the floor of the fourth ventricle, which is in agreement with the previously published statement of Cannon and Rapport (1921) that there are probably two centres which may cause a rise in blood pressure and an increased secretion of adrenaline, one situated in the hypothalamus and the other in the bulb (pons) Finally, it has been noted by Himwich and Keller (1930) that electrical stimulation of the region of the optic chiasma in cats under amytal anæsthesia was followed by a rise in the blood sugar level when the anæsthesia was light but not so when it was profound

In view of all this evidence pointing to the existence in the region of the hypothalamus of the cat's brain of a centre controlling, among other functions, the mechanism upon which the blood sugar level depends, it is difficult to explain why similar results were not obtained in our experiments on the rabbit. It cannot be accounted for by a paucity in the amount of glycogen in the liver—there was, for example, over 10 per cent of this in the liver of rabbit No. 132, and it cannot be attributed to anæsthesia, since the animal, being decerebrated, the effects of this had passed off several hours before most of the samples of blood were taken for analysis Moreover, the high degree of hyperglycæmia which invariably developed under identical conditions of

Table V —Behaviour of Blood Sugar following Decerebration in Position III

No of experiment	Normal B S	B S immediately after decerebration	B S at hourly intervals after decerebration			
			1	2	3	4
	per cent	per cent	per cent	per cent	per cent	per cent
58	0 117	0 135	0 117	0 151	0 193	0 249
66	0 119	0 167	0 274	>0 375	>0 375	>0 375
69	0 080	0 157	0 202	0 265	—	—
75	0 102	0 152	0 111	0 152	0 265	0 311
78	0 105	0 203	0 313	>0 375	0 320	0 201
79	0 142	0 151	0 114	0 178	0 231	0 241
82	0 098	0 182	0 141	0 182	0 174	0 184*
83	0 113	0 161	0 265	0 306	0 327	0 310
84	0 110	0 154	0 186	0 225	0 279	>0 450
87	0 131	0 187	0 173	–	—	0 260
91	0 116	0 136	0 186	0 238	0 297†	0 306
93	0 107	0 150	0 193	0 258	0 338†	0 236
95	0 088	0 149	0 154	0 203	0 300†	0 303
97	0 100	0 150	0 156	0 207	0 250	0 235
99	0 100	0 147	0 175	0 224	0 271	0 287
124	0 101	0 161	0 164	0 268	0 322	0 352
126	—	0 144	--	0 260‡	–	0 404§
130	0 107	0 150	0 222	0 256	0 301	0 346
131	0 133	0 172	0 314	--	—	—
142	0 140	0 171	0 254	—	-	
143	0 101	0 116	0 216	—	--	-
146	0 106	0 136	0 200	0 246	0 280	
149	0 106	0 141	0 164	0 212	—	0 287
Average	0 110	0 155	0 195	0 241	0 286	0 291
Difference	0 045	0 040	0 046	0 045	0 005	

* Large hæmorrhage † Ergotamine after † ‡ + 1½ hours § + 3½ hours

anæsthesia, etc , when decerebration was performed at the level of the pons (*vide infra*), rules out any chance of experimental error on account of anæsthesia.

Very different results were obtained when decerebration was performed in the region of the pons (region III) in a series of 22 experiments (Table V) decided hyperglycæmia developed in 21 within 4 hours after decerebration, the only exception being in an animal in which a very extensive pontine hæmorrhage was present If we take the average of all the experiments, it can be seen that the rise in blood sugar percentage is a very steady one and that it begins with the first sample of blood to be removed after completing the decerebration The interval elapsing between decerebration and collecting this sample varied slightly, owing to irregularity in the rate of bleeding, but it never exceeded 5 minutes The operative procedure *per se* cannot be held entirely accountable for this initial hyperglycæmia, since it was inconspicuous in the experiments of regions I and II, Plate 4 It would appear, therefore, that the nerve impulses which are responsible for the hyperglycæmia occur immediately decerebration is performed, and that these initial impulses are more effective than subsequent ones, since they can act in face of the inhibiting influence of amytal As we shall see later, the blood sugar does not rise above the level attained immediately after decerebration when the animals are kept deeply anæsthetised by repeated injections of amytal

The average blood sugar percentage continued to rise at about the same rate until between the third and fourth hours following decerebration when the rate of increase usually fell off considerably

Decerebration further back than the pons (in region IV) was performed in 10 experiments without any of them showing a significant increase in the percentage of blood sugar (Table VI) The only noteworthy change was that observed in the blood removed immediately after the decerebration Taking the average of all the experiments of this table, there was an immediate increase of 0 028 mg per cent over the predecerebration value, and since this increase is less than that observed for the experiments in region III but above that for those of regions I and II, we attribute it to unavoidable irritation of the pons during the operation

Table VI —Behaviour of Blood Sugar following Decerebration in Position IV

No of experiment	Normal B S	B S immediately after decerebration	B S at hourly intervals after decerebration			
			1	2	3	4
	per cent	per cent	per cent	per cent	per cent	per cent
86	0 114	0 130	0 097	0 106	0 112	0 104
92	0 108	0 124	0 125	0 143	0 170	0 211
104	0 104	0 138	0 116	0 104	0 102	0 113
111	0 109	0 134	0 138	0 141	0 141	0 141
113	0 116	0 172	0 188	0 172	0 184*	—
114	0 085	0 136	0 116	0 118	0 141	0 143
116	0 165	0 168	0 148	0 165†	0 079	0 099
141	0 092	0 080	0 080	—	0 092	0 075
147	0 109	0 145	0 116	0 128	0 112*	—
148	0 096	0 138	0 115	*0 140	0 185	—
Average	0 109	0 137	0 123	0 135	0 131	0 126

* Circulation poor † Insulin injected after

The almost unfailing certainty with which marked hyperglycæmia could be induced by decerebration at the pons, although not at other levels of the cerebro-spinal axis, raised the question whether like results would be obtained by puncturing it with a dissecting needle through the floor of the fourth ventricle under conditions of anæsthesia, etc., similar to those used in the decerebration experiments Although the experiments of this nature were really performed prior to those in which decerebration was employed, they would seem to fit in here The rabbits after ordinary feeding were injected intravenously with amytal and when deeply anæsthetised, the occipito atlantoid membrane was opened and the floor of the fourth ventricle punctured in various positions, as shown in fig 2, the direction of puncture being usually forward The wound was then closed and the animal allowed to recover from the amytal, samples of blood being removed for analysis at hourly intervals. The animals usually recovered from the anæsthesia in about 1 hour The results are shown in Table VII

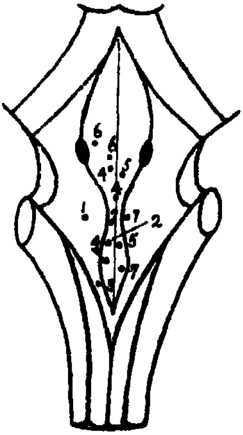

FIG 2—Outline drawing of floor of fourth ventricle in rabbit's brain, showing position of punctures

1	B S in 4 hours	0 171 per cent *
2	,,	0 259 ,,
3	,,	0 161 ,, *
4	,,	0 294 ,,
5	,,	0 110 ,,
6	,,	0 102 ,,
7	,,	0 106 ,,

* In 5 hours

Marked hyperglycæmia developed in two of the experiments (Nos 51 and 53) and in both of these post-mortem examination showed that the puncture had passed through the pons There was a moderate rise in blood sugar percentage in two of the remaining experiments (Nos 48 and 52) but, unfortunately, there is no post-mortem record to show what structures were involved in the punctures To judge from their positions on the floor of the ventricle it is

Table VII.—Blood Sugar percentages at hourly intervals after recovery from Amytal Anæsthesia during which Puncture of the Floor of the 4th Ventricle had been performed

(The exact position of the punctures is shown in fig 2)

No of experiment.	On chart.	Before amytal	After amytal + piqûre.	1	2	3	4	5.	6	Blood lactic acid *	Remarks
48	1	0 133	0 171	0 166	0 166	0 206	0 137	0 171	0 190	++	—
51	2	0 104	0 121	0 129	0 143	0 141	0 259	0 311	0 309	—	Pons involved, much bleeding
52	3	0 161	0 163	0 200	0 196	0 174	0 192	0 161	0 147	++	—
53	4	0 116	0 149	0 198	0 281	0 314	0 294	0 234	0 130	Nil	Pons involved. Liver glycogen 0 62 per cent
54	5	0 106	0 102	—	0 107	0 101	0 110	0 110	0 113	+	2 punctures Bleeding Pons involved on both sides Liver glycogen 1 18 per cent.
55	6	0 107	0 106	0 114	0 100	0 100	0 102	0 104	0 104	Nil	2 punctures. Liver glycogen 1 45 per cent.
57	7	0·081	0 082	0 092	0 099	0 116	0 106	0 099	0 076	Nil	3 punctures Liver glycogen 1 2 per cent.

* "Nil" indicates that no significant change occurred, *plus* signs, the extent to which it increased.

unlikely that the needle passed through the pons in either experiment In the remaining three experiments, the blood sugar percentage underwent no change, although in at least two of them (54 and 55), the pons was apparently involved

We do not know whether piqûre of the pons through the floor of the fourth ventricle with an instrument larger than the needle used by us would cause hyperglycæmia That it probably would, is indicated by the results published by Hiller (1930) who, in order to avoid the confusing effects of ether anæsthesia, did not perform the piqûre until 5 hours after withdrawing the anæsthetic, the floor of the fourth ventricle having been exposed while the animal was under its influence He found that piqûre of the medulla in the region of the dorsal nucleus of the tenth nerve was without significant effect on the blood sugar level, whereas piqûre in the pons and cerebellum, in the region of the vestibular nuclei, invariably caused marked hyperglycæmia These results are contrary to those of Brugsch, Diesel and Lewy (1920) which seemed to show that the diabetogenic centre is located in the dorsal nucleus of the vagus In this particular, our results definitely confirm those of Hiller It is interesting to note also that Cannon and Rapport (1921) concluded that a centre having an influence over the internal secretion of adrenaline may be situated in the bulb

It is scarcely necessary here to review the numerous other researches in which piqûre of the floor of the fourth ventricle has been performed In very few of them is the published information sufficient to tell exactly what structures were involved One gathers from several of the papers that there was an element of uncertainty as to whether the piqûre would or would not induce hyperglycæmia In the light of our results it is most likely that involvement of the pons rather than, as was supposed, a possible shortage of the percentage of glycogen in the liver, was the deciding factor As far as we can find, the nearest approaches to accuracy, in describing the position on the floor of the fourth ventricle at which the puncture was made, are in the papers by Stewart and Rogoff (1918) and Banting, *et al* (1923), and in them it is shown to have been between 4 and 8 mm above the tip of the calamus scriptorius But this is not sufficient information from which to determine whether the pons was involved in the pathway of the puncture. When Claude Bernard's instructions (1855) are accurately followed, there can be little doubt that the pons is involved in the puncture, so that " diabetes " occurs. This method consists in puncturing through the skull with a stylet, having at its end a cutting edge 2 mm wide The stylet is pushed through the skull just in front of the occipital protuberance and is directed in a line running from this to the external auditory meatus, injuring the cerebellum and often causing considerable hæmorrhage accompanied by violent convulsive movements. This renders the method useless when information is sought concerning possible changes in the metabolism of glycogen, lactic acid, etc, resulting from the piqûre When Eckhard's (1869) method is used exactly according to the directions contained in the original paper there is little doubt that the pons is involved in the puncture After opening the occipito-atlantoid membrane, the puncturing instrument is passed forward parallel to and a little above the floor of the fourth ventricle until its point is near the upper end when the handle is raised and the point pushed through the floor

Since the lesions in the pons in our experiments have in most cases extended the whole width of this structure, it is not possible to give the exact position of the centre In several experiments, as in all those of region IV, the corpora quadrigemina and the region between these bodies and the pons were involved without any hyperglycæmia occurring On the other hand, when the pons was punctured after exposing the fourth ventricle by opening the occipito-atlantoid membrane, the portions bordering on the corpora quadrigemina were not involved but, nevertheless, hyperglycæmia sometimes occurred These results indicate that the centre cannot be situated in the dorsal portions of the pons and the probability is that it resides in certain of the nerve cells of the tegmentum Hiller gives the centre as involving the region of the nucleus of the eighth nerve, its connections with the cerebellum and the cerebellum itself, and he states that rotation of a normal rabbit on a turntable for 5 minutes raised the percentage of blood sugar more than did piqûre of the nucleus of the tenth nerve While there is no doubt that the eighth nerve nuclei were involved in our decerebrations at the pons, there were several experiments in which the cerebellar attachments and the cerebellum itself were involved without any hyperglycæmia occurring, so that in this regard our results do not support Hiller's conclusions

It was considered by Claude Bernard that the irritation set up by the piqûre, rather than the destruction of some centre, or pathway, is responsible for the hyperglycæmic effects which follow This seems to us to be correct The only alternative explanation is that some pathway proceeding from a higher centre having an inhibitory influence on the mechanism, or mechanisms, concerned with the regulation of the blood sugar level is severed, but evidence that such an explanation is untenable is to be found in our results which show that no hyperglycæmia followed when the section was made *immediately* in front of the pons

The interest which has recently been taken in the possibility that the diabetic centre really resides in the hypothalamus led us to perform a large number of experiments in which this region was included in the decerebrations. In relatively few of these did we find hyperglycæmia to occur and when it did so it was usually much less pronounced than when the pons was involved. We cannot deny that a centre capable of influencing the blood sugar level may exist in this region, but we do not accept the evidence so far recorded in the literature as showing that the *chief* centre is here. It is possible that there are various regions of the brain provided with similar centres.

Summary

(1) Puncture of the floor of the fourth ventricle of the brain in rabbits having an average amount of glycogen in the liver and muscles has no effect on the blood sugar level while the animals are under the anæsthetic influence of "luminal" or "amytal".

(2) Under similar conditions of anæsthesia, injection of adrenaline results in hyperglycæmia, but mechanical asphyxia or ether is without effect on the blood sugar level, and lesions of the brain, such as decerebration or injury in the region of the hypothalamus, only occasionally cause it to rise.

(3) When decerebration is performed in rabbits during the short period of anæsthesia induced by the intravenous injection of amytal, marked hyperglycæmia supervenes within 2 hours, provided that the pons is involved. Decerebration anterior or posterior to this region seldom has any effect on the blood sugar level, except when it involves a narrow portion of the medulla adjacent to the pons.

DESCRIPTION OF PLATE

Plate 4.—Photographs of brains of rabbits decerebrated in positions I and II.

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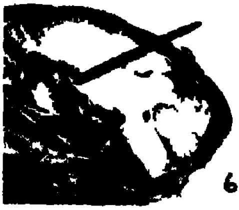
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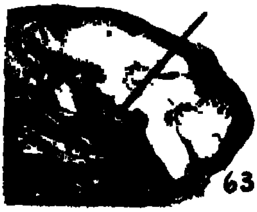
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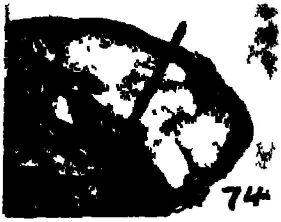
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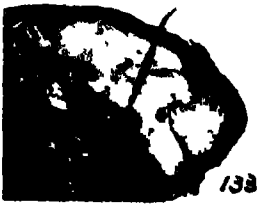
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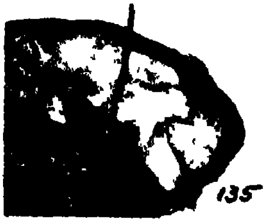
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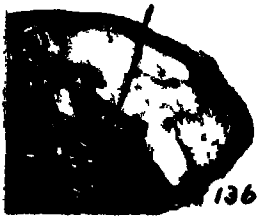
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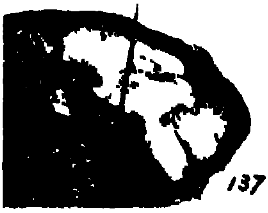
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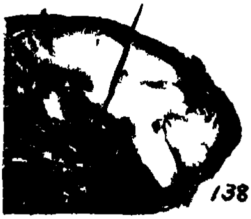
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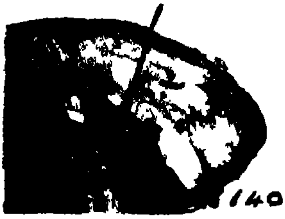
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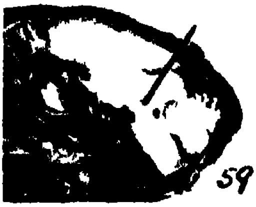
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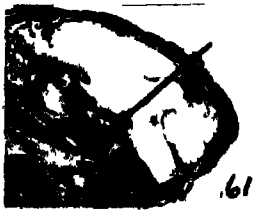
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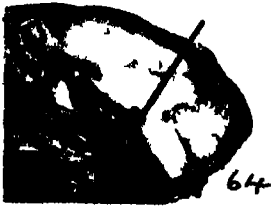
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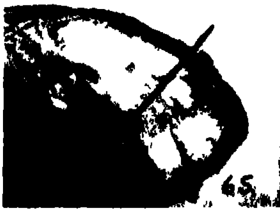
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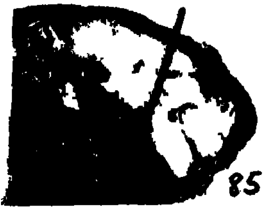
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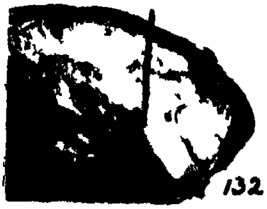
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Studies in the Nervous Control of Carbohydrate Metabolism
II - *The Chemical Changes set up in the Body during Decerebration Hyperglycæmia*

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The pronounced hyperglycæmia which follows decerebration at the level of the pons in rabbits may be due to increased production of glucose out of glycogen, to suppression of the utilisation of glucose by the tissues or to excessive new formation of glucose (gluconeogenesis) out of protein and fat

To ascertain which of these functions is involved, we have investigated the changes in the percentages of glycogen in the liver and muscles and of lactic acid and non-protein nitrogen in the blood produced by decerebration, and we have observed its effect on the respiratory metabolism

It was hoped, by a comparison of these changes with those produced by injecting adrenaline into normal rabbits, to obtain decisive evidence showing whether or not increased secretion of this hormone from the adrenal glands is the essential cause of the hyperglycæmia resulting from pontine decerebration These observations have been conducted on animals in varying states of nutrition and after experimental interference with the continuity of the possible nervous pathways through which the diabetogenic impulses might be conducted

Methods

The methods used for the various determinations have been standard ones and it is necessary here to refer only to certain details of procedure with regard to measurement of the behaviour of glycogen in the liver and muscles and of the respiratory exchange

The Glycogen of Muscles and Liver —Immediately after inserting the tracheal cannula, a stout cord was placed around the right leg just above the knee-joint, and amputation performed by a hatchet while the cord was tightened By this procedure practically no blood was lost The amputated leg was allowed to fall into a freezing mixture and when thoroughly cold was skinned and the muscles cut off and placed in hot 60 per cent KOH solution There was seldom more than a slight twitch of the muscles when the leg was put in the freezing mixture and of course, none at all when the muscles were excised and placed in the potash This method was adopted in light of the results obtained by Anderson and Macleod (1930) and after the attempt had been made in some of the earlier of the present experiments, to dissect out particular muscles The abdomen was opened in the mid-line and a flat ligature (shoe-lace) tied around the small central lobe of the liver, the tip of which was cut off and immediately placed in hot 60 per cent KOH solution

The decerebration was then performed, as described in the previous paper, and amytal was not given again until the end of the experiment when a second intravenous injection was made to prevent reflex twitching of the muscles The left leg and another portion of liver were then removed for analysis

The Respiratory Observations —The rabbit was placed in a metal box containing about 4000 c c of water at 35–37° C This box was enclosed in a larger one, the space between being packed with broken cork The box was covered by a glass lid and sometimes wrapped in a thick blanket The tracheal cannula was connected with the Olmsted-Taylor pump by means of which artificial respiration was maintained, the expired air being collected for periods of 15 minutes each at hourly intervals in a 20 litre rubber bag The minute volume of air was 700 c c , the rate of breathing (*i e* , of the pump) 30 per minute and the respiratory volume of each breath therefore approximately 23 5 c.c. These conditions were kept constant in all the experiments (unless otherwise noted) and only very occasionally did any of the animals show spontaneous breathing After metering the air which had been collected, a sample was carefully analysed in a modified Haldane apparatus The temperature of the animal was read, at least hourly, on a thermometer inserted among the muscles of the thorax and the temperature of the bath, after stirring, on thermometers placed at each end of it

Results

Those for glycogen and lactic acid are collected in Tables I, II and III In all of the experiments grouped in Table I, decerebration was performed at the pons and in these the concentration of sugar in blood removed 4 hours later reached or exceeded 0 18 per cent In those of Table II, decerebration was performed at other levels and the blood sugar percentage did not change In those of Table III, the influence of various other experimental conditions is shown The changes observed in the concentration of the lactic acid of the blood and the glycogen of the liver and muscles were as follows —

The ***blood lactic acid*** immediately after decerebrating was fairly uniform in the different experiments and averaged 0 039 mg per cent After 2 hours its behaviour was irregular if we allow ± 5 mg per cent for experimental error, it remained unchanged in four of the fifteen experiments in which it was measured, decreased in five and increased in six After 4 hours, on the other hand, the lactic acid increased decidedly in all but two of the experiments (Nos 100 and 124), and in those in which it was observed at a later period the increase became very marked It may be of some significance that in one of the exceptional experiments, the glycogen percentage increased in the muscles and in the other it fell to a moderate degree only If we take the average of all the experiments, it is clear that the increase in lactic acid occurred mainly after the second hour Two possible causes for this delay in the rise in lactic acid have to be considered (1) the remaining effects of the amytal may retard its increase at first, and (2) failing circulation may stimulate its production later Amytal alone had no influence on the lactic acid level of normal rabbits, as is shown in the results of Table III, and this conclusion agrees with that of other workers (Hinsey and Davenport, 1929) But this does not necessarily indicate that this narcotic has no effect in retarding or preventing the accumulation of lactic acid in the blood after decerebration It would be necessary to observe the behaviour of the lactic acid after decerebration under local anæsthesia, before deciding whether the amytal anæsthesia was responsible for retarding the increase in lactic acid in our experiments It may be of some significance in this connection, that amytal does not interfere with the rise in lactic acid following injection of adrenaline (Cori and Cori, 1928) Nevertheless, it retards the breakdown of glycogen in the muscles in normal animals (Hinsey and Davenport, 1929, Eggleton and Evans, 1930).

Table I —Lactic Acid of Blood and Glycogen of Liver and Muscles after Pontine Decerebration.

No of experiment	Body weight (kg).	Nutritional state	B S 4 hours after (per cent).	Duration of experiment after decerebration (hours).	Lactic acid in blood (mg per cent.).				Glycogen (per cent.).				
					After op.	2 hrs.	4 hrs.	End	In muscles.		In liver		
									Begin.	End.	Begin.	End	
58	—	Fast	0 25	—	43 0	22 0	54 0	130 0	—	0 37	—	0 73	G. of muscles is average of tib ant. and gracilis.
75	2 85	,,	0 31	5 0	49 0	105 0	265 0	388 0	0 43	0 22	1 10	0 91	
78	2 30	,,	0 20*a*	4 5	73 0	66 0	96 0	101 0	0 36	0 26	0 62	0 79	*a* B S. was 0 37 at 2 hours
82	2·15	,,	0 18	4 0	45 0	33 0	75 0	—	0 54	0 40	0 57	1 69	
83	3 09	,,	0 31	4 0	42 0	32 0	62 0	—	0 35*b*	0 41*b*	0 16	0 41	*b* Much twitching
84	1 24	,,	0 35	4 0	62 0	66 0	—	—	0 21	0 10	1 29	0 12	
99	1 46	,,	0 29	4 0	19 0	59 0	159 0	—	0 40	0 19	0 69	0 16	
100	2 02	,,	0 26	4 0	41 0	20 0	23 0	—	0 18	0 26	0 95	0 47	
118	1 52	,,	0 31*c*	2 0	48 0	86 0	—	—	0 21	0 12	0 12	0 03	*c* 2 hours after cut.
119	1 77	,,	0 20	2 0	—	24 0	—	—	0 15	0 08	0 32	0 33	Do
124	2 25	,,	0 35	4 0	14 0	21 0	26 0	—	0 34	0 26	2 44	1 06	
146	1 50	,,	0 28*d*	3 0	—	—	—	—	0 37	0 25	1 93	1 57	*d* 3 hours after cut
79	1 74	Fed	0 24	4 5	43 0	40 0	—	117 0	0 70	0 58	6 99	4 32	
91	1 97	,, *e*	0 31	5 0	24 0	85 0	107 0	95 0	0 72	0 64	7 66	2 33	*e* Ergotamine
93	1 22		0 24*f*	4 0	22 0	85 0	42 0*g*	—	0 62	0 16	1 25	0 56	*f* B S. was 0 34 at 3 hours.
95	2·60	,	0 30	4 0	40 0	41 0	125 0*e*	—	0 57	0 56	7 25	2 36	
97	3 25	,,	0 26	4 0	34 0	29 0	55 0	—	0 77	0 71	5 75	3 45	*g* Heart blood.
149	1 17	,, *h*	0 29	3 5	—	—	—	—	0 64	0 39	9 55	4 33	*h* Stock diet.

Note.—The food consisted of carrots, sugar and maize, and it was not withdrawn from the cage before removal of the animal

Table II.—Lactic Acid of Blood and Glycogen of Liver and Muscles after Decerebration elsewhere than in the Pons, and not showing Hyperglycæmia

No. of experiment.	Nutritional state.	B.S. 4 hours after decerebration or at death (per cent.)	Duration of experiment after decerebration (hours)	Lactic acid in blood (mg. per cent.).				Glycogen (per cent.).			
				After op.	2 hours.	4 hours	End	In muscles. Begin.	In muscles. End	In liver Begin.	In liver End.
					Position I						
62	Fast	0·098	6·0	40·0	23·0	19·0	28·0	—	—	0·92	—
74	,,	0·11	7·5	—	29·0*	32·0	33·0	—	—	—	—
136	,,	0·16	4·0	—	10·0	8·0	9·0	0·64	0·58	3·10	2·8
137	,,	0·15	4·0	—	8·0	9·0	11·0	0·62	0·51	2·58	1·5
138	,,	0·09	3·7	—	16·0	14·0	—	0·40	0·25	0·16	0·14
139		0·13	3·5	—	8·0†	8·0‡	—	—	—	—	—
					Position II						
59	Fast	0·13	3·0	—	49·0	—	57·0	—	—	—	—
61	,,	0·15	8·0	—	43·0	33·0	52·0	0·24	—	0·32	—
85	Fed	0·11	7·5	30·0	45·0	—	80·0	0·12	0·17	1·21	1·40
132	Fast	0·15	4·0	13·0*	17·0	46·0‡	—	0·61	0·53	10·4	9·01
					Position IV						
104	Fed	0·11	4·0	40·0	39·0	50·0	—	0·17	0·32	0·53	1·36
111	Fast	0·14	4·0	22·0	25·0	24·0	—	0·29	0·40	0·94	1·35
113	,,	0·18	3·0	42·0	38·0	—	52·0	0·04	0·06	0·57	0·32
114	,,	0·14	4·0	—	15·0	23·0	—	0·11	0·10	1·12	0·36
116	,,	0·16	2·0	—	22·0	—	—	—	—	—	—
141	,,	0·07	4·0	—	—	—	—	—	—	0·16	0·17
86	Fed	0·10	4·5	43·0	56·0	—	71·0	0·27	0·65	1·96	1·50
147	,,	0·11	3·5	—	—	—	—	0·51	0·44	—	—
148	,,	0·18	3·5	—	—	—	—	0·74	0·73	8·45	8·15

* In 1 hour † In 1½ hours ‡ In 3 hours

Table III —Lactic Acid of Blood and Glycogen of Liver and Muscles

No. of experiment.	B S. 4 hours after giving amytal (per cent.)	Duration of experiment (hours).	Lactic acid in blood (mg per cent.).					Glycogen (per cent).			
			Normal.	1 hour	2 hours	3 hours	4 hours.	In muscles		In liver	
								Before	After	Before.	After
I —In Animals receiving Amytal Intravenously											
68	0 09	5 0	27 0	20 0	21 0	37 0	32 0	—	0 26	—	0 48
89	0 13	4 5	19 0	16 0	13 0	17 0	20 0	0 41	0 36	1 17	1 23
90	0 12	4 5	—	22 0	—	32 0	26 0	0 59	0 53	3 01	2 62
II —In Animals showing Hyperglycæmia although Decerebration was not in Pons											
73*	0 24	5 0	28 0	26 0	—	32 0	35 0	—	—	—	—
135*	0 39	4 5	22 0	36 0	—	76 0	106 0	0 85	0 59	11 00	7 56
III —In Animals after Adrenaline (and Amytal)											
80>	0 34	4 0	45 0	—	—	—	130 0	0 55	0 23	2 26	0 22

* Section olfactory bulbs followed after 3 hours by destruction of tuber cinereum. Cut at tuber cinereum.

In the experiments in which decerebration was performed at levels other than the pons (Table II), the lactic acid was somewhat irregular in its behaviour After 2 hours it remained near the normal level in all of the six experiments in which the decerebration was in position 1, it increased in three of the four experiments when the decerebration was in position II, and in one out of six when it was in position IV After 4 hours or more, there were no noteworthy changes in lactic acid, except that a moderate increase usually occurred in the experiments in which decerebration was in positions II and IV These results show, among other things, that the marked lactacidæmia which was present 4 hours after pontine decerebration cannot have been due mainly to failing circulation (and consequent anoxæmia of the muscles)

Cori and Cori found after injecting adrenaline into rabbits that the changes which occurred in the concentration of blood sugar ran parallel with those in lactic acid In the present investigation this parallelism was not a close one, as may be seen by comparison, either of the hour to hour behaviour of the two substances in individual experiments or of the average level attained in all of them at a definite period (4 hours) after decerebration As typical experiments, we may take No 99 representing one in which the liver glycogen was low, because of fasting, and No 97 representing one in which it was high, because of special feeding

Experiment No	Time after decerebration (min)	Blood sugar (per cent)	Lactic acid (mg per 100 c c)	Remarks
99	Normal	0 100	22 5	Glycogen percentage in liver at start, 0 685
	Immediate	0 147	19 0	
	1 hour	0 175	-	
	2 hours	0 224	59 0	
	3 ,,	0 271	60 0	
	4 ,,	0 287	159 0	
97	Normal	0 106	42 5	Glycogen percentage in liver at start, 5 75.
	Immediate	0 150	33 5	
	1 hour	0 156	-	
	2 hours	0 207	28 5	
	3 ,,	0 250	25 0	
	4 ,,	0 235	55 0	

If we arrange the results of all the experiments of Table I in order of increasing concentrations of blood sugar at 4 hours after decerebration, those of lactic acid run as in table on following page

In seeking for the cause of the increases in sugar and lactic acid in the blood, we naturally turn to the behaviour of the glycogen of the liver and muscles,

Blood sugar was	Lactic acid	Blood sugar was	Lactic acid
per cent	mg per cent	per cent	mg per cent
0 18	75	0 29	159
0 20	96	0 30	125
0 24	85	0 31	265
0 25	54	0 31	62
0 26	23	0 31	107
0 26	55	0 35	26

and the results which demonstrate this are included in the Tables I to III In some cases the animals were deprived of food (stock diet) during a period of 12 to 24 hours before the experiment, in others, they were given food rich in carbohydrate

The Liver Glycogen -In the animals which were fasted for 16 hours or longer preceding the experiment, the glycogen of the liver at the beginning varied between 0·12 and 2·5 per cent and there was no obvious relationship between the duration of the fasting period and the glycogen percentage The glycogen of the liver increased decidedly in three experiments (Nos 78, 82 and 83), it remained practically unchanged in three (Nos 75, 118 and 119) and decreased in the remaining five (Nos 84, 99, 100, 124 and 146) It is significant that in five of the six instances in which the glycogen either increased or remained constant, it was below 0 7 per cent to start with, showing that none of the increase in blood sugar can be attributed to excessive production of glucose out of the glycogen stores of the liver Even when, as in other experiments, some of the extra sugar may have been so derived, the amount of this was far short of that necessary to account for the hyperglycæmia, for example, the maximal change in any experiment of this group was from 2 44 per cent. to 1 06 per cent (No 124), a difference of 1·38 per cent which, assuming the liver to be 5 per cent of the body weight—which was 2 25 kg -gives a total of about 1 55 gm of glucose Injected continuously over a period of 4 hours into a rabbit of similar weight, this amount of glucose would not raise the blood sugar curve to anything like the extent observed after decerebration. Thus Cori and Cori (*loc cit.*) found that it required over 2 gm glucose per kilogram and hour injected intravenously to raise the blood sugar percentage of normal rabbits to about 0 300 In experiment 124 the rate at which sugar was lost from the liver equals 0 17 gm per kilogram and hour

In concluding that very little, if any, of the extra blood sugar in many of the experiments can have come from the initial glycogen stores of the liver, we do not mean to imply that we regard changes in the glycogen percentage of the liver as bearing any definite relationship to the amount of glucose which this organ may deliver into the blood On the contrary, we consider that glycogen in the liver exists in two physiological states, one a storage form built up from preformed glucose and another, a gluconeogenic form synthetised from protein, lactic acid, etc , and not, as a rule, stored up to any extent The glycogenic function of the liver, at least in so far as the gluconeogenic form of glycogen is concerned, must be thought of in the dynamic rather than the static sense, and glucose may be produced from this glycogen as quickly as it is being formed Observed changes in percentage of glycogen are therefore no measure of the amount of glucose which may have been formed from it or, conversely, of the extent to which glycogen formation may have been going on Later on we will discuss more fully these aspects of our problem

Turning to the experiments in which, as a result of special feeding, large percentages of glycogen were present in the liver to start with (Nos 79, 97 and 149), we see that a much higher proportion of the extra blood sugar may have come from this source In one case, for example (No 149), the percentage of glycogen fell during the experiment from 9 55 to 4 33, a difference of 5·22, thus accounting for 3 05 gm of glucose (since the animal weighed 1·17 kg). The experiment lasted 3 5 hours, so that about 1 gm of glucose per kilogram and hour was derived from the preformed glycogen of the liver, an amount which, if injected intravenously, would, to judge from Cori's results, have raised the blood sugar percentage to about half the height actually observed in the experiment The results therefore show that even when the preformed glycogen of the liver is very high to start with, its breakdown does not furnish all of the extra sugar which appears in the blood after decerebration

With regard to the behaviour of the liver glycogen in cases in which decerebration, being elsewhere than at the pons, was not followed by hyperglycæmia, it will be seen that out of eight experiments (Table II) in which the animals were fasted, the glycogen percentage remained practically unchanged or it increased in six (Nos 136, 138, 104, 111, 113 and 141) and it decreased decidedly in two (Nos. 137 and 114) This result, therefore, does not differ essentially from that obtained after pontine decerebration In only two out of four experiments (Nos 132 and 148) in which fed rabbits were used, was the initial glycogen in the liver very high, and in these it scarcely became less as a result of the decerebration In two other fed rabbits (85 and 86), the initial glycogen was unfortunately not very high, but in neither of them did the decerebration cause it to change significantly It is probably safe to conclude that the small changes in liver glycogen observed in this group of experiments were no different from what they would have been had no decerebration been performed This conclusion is supported by the results collected in Table III, from which it can be seen that very little change occurred in the percentages of glycogen of muscles and liver, or of the sugar and lactic acid of the blood, as a result of giving one anæsthetic dose of amytal intravenously and then allowing the animal to remain quiescent for 4 hours

The Muscle Glycogen—In nine out of the eleven experiments on fasted rabbits shown in Table I, pontine decerebration caused some reduction in the percentage of glycogen in the muscles In one of the exceptions (No 83) there was a doubtful increase (from 0 35 to 0 41 per cent *) and at the same time the glycogen of the liver rose from 0·16 to 0 41 per cent In this experiment hyperglycæmia was pronounced and lactacidæmia moderate in degree, so that unless carbohydrate was still being absorbed from the alimentary canal—which is unlikely since the animal had been fasted 48 hours—the extra sugar must have been produced by a process of gluconeogenesis In the other experiment in which an increase in muscle glycogen occurred (No 100) the liver glycogen decreased by about 0 5 per cent or *in toto* 0 3 gm (liver weighed 60 gm), which falls short not only of the amount which could be held accountable for the increase in sugar which occurred in the blood of this animal, but also for the glycogen which accumulated in its muscles (0 64 gm)

The average glycogen in the muscles to start with in the remaining nine experiments of this group was 0 334 per cent , and at the end, 0 210 per cent Assuming that the musculature of the rabbit is 40 per cent of the body-weight and that the leg muscles which were taken for analysis are representative, with regard to their glycogen content, of the body as a whole, then 0·5 gm of sugar per kilogram body-weight was, on a rough average, made available from muscle glycogen Injected intravenously over a period of 4 hours this amount of glucose would have had very little effect on the blood sugar level

If we select the experiment in which there was the greatest diminution of glycogen in both liver and muscles and calculate in terms of glucose the amount of glycogen which disappeared from these tissues during the 4 hours following decerebration, the results are as follows · (experiment 99), weight of rabbit 1·46 kg , weight of liver 33 4 gm Glycogen disappeared from muscles 0·21 per cent or *in toto* 1·22 gm , glycogen disappeared from liver 0·53 per cent or *in toto* 0 165 gm In all, about 1·385 gm glucose was thus set free, an amount which is far from being adequate to account for the hyperglycæmia observed in this animal

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* The muscles in this experiment twitched when put in the freezing mixture.

In no other experiment of this series can more than 1·5 gm of glucose be shown to have been set free by the decerebration It is quite clear from these results that the increase in the percentage of blood sugar which follows decerebration in previously fasted animals cannot, to any large extent, be attributed to mobilisation of the glycogen present in the liver and muscles to start with

On the other hand, in four experiments (Nos 91, 95, 97 and 149) in which the liver and muscles were heavily loaded with glycogen, the sugar derived from these sources along with that absorbed from the alimentary canal was probably sufficient to account for the extra sugar and lactic acid in the blood. In three of them the liver was very rich in glycogen and the muscles lost little of their stores of this material In two of the experiments ergotamine was injected after the hyperglycæmia had developed, but it is considered unlikely that this can have been responsible for the stability of the muscle glycogen

Finally, it is noteworthy in connection with the controls that the muscle glycogen percentage, which varied considerably to start with, underwent practically no change in four of them (Nos 136, 113, 114 and 148), increased in four (85, 86, 104 and 111), (three of these being again in animals decerebrated through the medulla) and decreased in four (137, 138, 132 and 147) It either remained unchanged or increased in amount in all but one of the seven cases of medullary decerebration

In three of the "controls" (85, 104, 111), the glycogen increased in both liver and muscles In two of these the animals had recently been feeding, so that the extra carbohydrate was probably absorbed from the intestine In the remaining one, however, the animal was fasted, so that gluconeogenesis must have been occurring The observation indicates that this process may become excessive in the absence of hyperglycæmia Table III also shows the behaviour of lactic acid and glycogen in the two exceptional experiments in which hyperglycæmia developed after decerebration in the region of the tuber cinereum, and in one in which adrenaline was injected The amounts of glycogen which disappeared from liver and muscles are probably sufficient to account for the extra sugar and lactic acid

Non-protein Nitrogen of the Blood.

In those experiments on fasted animals in which little, if any, decrease occurred in the glycogen stores as a result of decerebration, the possibility has to be considered that an excessive new formation of carbohydrate was taking place to account for the hyperglycæmia. In the event that protein was the material furnishing this sugar, its metabolism would become greater and it seemed possible that this might be revealed by measurement of the non-protein nitrogen of the blood, observations of the urinary excretion of nitrogen being impossible under the conditions of the experiment We give the results obtained when the decerebrations were in positions III and IV

Table IV —The Non-protein Nitrogen of the Blood following Decerebration

No of experiment	Condition of animal	Position of decerebration	B S in 4th hour after decerebration	N P N (mg per cent.)	
				Begin	End
145	Fasted 48 hours	IV	0 15	42 0	49 0
146	Fasted 24 hours	III	0 28	57 5	70 0
147	Fasted 12 hours	IV	0 11	49 0	49 0
148	Fed	IV	0 18	41 3	35 0
149	Fed	III	0 29	30 8	33 9

The greatest increase occurred in a fasted animal in which the hyperglycæmia was moderate in degree but, although this result is suggestive, it will be necessary, before drawing definite conclusions, to repeat the observations on animals from which the kidneys have been removed

The Respiratory Exchange —This was measured after decerebration in order to see whether the consumption of oxygen would increase, as after the injection of adrenaline into normal animals, or the respiratory quotient decrease, and so give evidence of a diminution in the relative amount of carbohydrate undergoing oxidation In connection with the former possibility, it is well known that injection of adrenaline is followed by an increase in the oxygen consumption of the fasting animal without there being any change in the respiratory quotient (Cori and Cori, 1928, Boothby and Sandiford, 1923) Some uncertainty arises in interpreting the results on account of the difficulty of obtaining suitable controls. A normal rabbit under amytal anæsthesia cannot be used for this purpose because its metabolism is depressed and when consciousness returns the animal becomes too restless to permit of respiratory observations by the Douglas bag method Neither can an animal be used in which the level of decerebration is anterior to the pons, because of the hyperexcitability of the reflexes The most suitable controls would probably be furnished by animals in which decerebration was performed through the medulla but, unfortunately, our experiments on this preparation came to grief In order to overcome these difficulties, we have used as controls the results obtained on the same animal prior to decerebration

In all experiments, therefore, two respiratory observations were made after the necessary preliminary operations had been completed under amytal anæsthesia. Approximately 1 hour elapsed between these observations, and at the end of this period decerebration was quickly performed It can be seen from the table of results (Table V) that the oxygen consumption fell slightly during the interval prior to decerebration, so that we will take the average of the two determinations as our controls. One hour was allowed to elapse after decerebration before again measuring the respiratory exchange

Pronounced hyperglycæmia developed in five out of the six experiments, and the average oxygen consumption (Table VI) rose decidedly in two of the experiments (Nos 156 and 158), rose slightly in another, and in the remainder either remained steady or fell a little The rise, when it occurred, was already evident in 1 hour after decerebration and became more marked in 2 and 3 hours , subsequently the O_2-consumption showed a tendency to fall The average respiratory quotient did not undergo any significant change, except in one of the experiments in which the O_2-consumption was unaffected by decerebration The results show, with one exception (No. 158) that decerebration causes comparatively little alteration either in the consumption of O_2 or the respiratory quotient The stimulation of metabolism, when it occurred, may possibly have been due to an increased internal secretion of adrenaline, but it does not seem likely that this would happen in some cases and not in others There is also no evidence to indicate that the metabolism of carbohydrates became suppressed

We are, therefore, at a loss to account for the exact source of the extra sugar responsible for causing the hyperglycæmia following pontine decerebration in animals in which, owing to previous fasting, no absorption of glucose from the alimentary canal could have been occurring But it must be remembered that we have no means of telling exactly how much extra glucose is necessary to bring about the degree of hyperglycæmia which was observed In considering whether the glucose might have arisen from the breakdown of glycogen in the liver and muscles, we have assumed the quantity necessary for this purpose to be that which must be injected intravenously into a normal rabbit in order to produce a comparable hyperglycæmia In such a case, however, it is certain that some of the injected glucose is deposited as glycogen and that some is oxidised (specific dynamic action) Since neither of these processes for disposing of excess of glucose may have been operating in the decerebrated animals, the above comparisons are probably of little value, and it is possible that the degree of hyperglycæmia which developed in them did not bear any

Table V.—The Respiratory Exchange after Pontine Decerebration

No of experiment.	Body weight (kg).	B.S. per cent at end	Feeding	Position of decerebration	Body temperature		Oxygen consumption in c.c. per kg and hour and R.Q. at approximately hourly intervals													
							Before decerebration		After decerebration											
							1 hour		Immediately		1 hour		2 hours		3 hours		4 hours		5 hours	
					Beginning	End	O₂	R.Q.	O₂	R.Q.	O₂	R.Q.	O₂	R.Q.	O₂	R.Q.	O₂	R.Q.	O₂	R.Q.
							c.c.		c.c.		c.c.		c.c.		c.c.		c.c.		c.c.	
156	1.5	0.35	Fast	III	36.8	36.4	452	0.70	386	0.76	447	0.77	501	0.78	514	0.70	487	—	—	—
158	2.7	0.27	,,	III	37.5	37.2	307	0.86	249	0.89	354	0.69	405	0.77	393	0.86	340	0.98	—	—
159	2.9	0.30	,,	III	38.1	36.0	326	0.73	267	0.84	278	0.99	292*	0.83	307	0.89	273	0.86	254	0.94
160	1.5	0.33	Fed	III	36	35.8	415	0.88	379	0.96	264	0.90	361	0.92	415	0.92	—	—	—	—
162	1.7	0.35	,,	III	37	37.2	402	0.91	379	1.0	411†	0.89	352	1.0	390	0.93	374	0.95	376	0.95
163	2.3	0.20	,,	III	39	38.7	422	0.84	386	0.82	401	0.86	416	0.85	445	0.88	432	0.85	394	0.92

* Animal restless, decerebration repeated somewhat further back.
† The intervals after decerebration are 30 minutes, 1½ hours, 2½ hours, etc.

Table VI—Average Consumption of Oxygen and Average R Q before and following Pontine Decerebration

No of experiment	Before decerebration		After decerebration O_2 c c per kg and hour	Percentile change	R Q
	O_2 c c per kg and hour	R Q			
156	420	0 73	482	+14 2	0 75
158	278	0 87	373	+34 1	0 82
159	291	0 78	287	-	0 89
160	397	0 91	347	−12 6	0 91
162	390	0 95	382	− 0 2	0 94
163	404	0 83	423	+ 4 7	0 86

quantitative relationship to the changes occurring in the total amount of glucose in the body.

In this connection it seemed of interest to ascertain whether some difference could be detected in the glycogen-forming powers of liver and muscles in animals decerebrated at the pons and in those decerebrated at other levels We therefore studied the effect of injecting glucose This has also been done by Evans, Tsai and Young (1931), in decapitate cats without their finding that the injection appreciably altered the rate of glycogen formation in the liver or caused glycogen to be formed in the muscles

Table VII—The Effect of Glucose Injections in Decerebrated Animals (The injections were given immediately after the first hour following decerebration)

No of experiment	Position of decerebration	Blood sugar (per cent)				Glycogen (per cent)			
		Hours after decerebration				Muscles		Liver	
		1	2	3	4	Begin	End	Begin	End.
127	IV	0 08	0 22	0 23	-	0 11	0 10	0 03	0 04
128	IV	0 16	0 40>	0 49>	0 40	0 40	0 36	2 48	1 87
133	II or III	0 20>	0 42>	0 42>	0 49	0 47	0 48	3 53	6 28
134	III	0 20	0 51	0 52	—	0 62	0 51	0 97	0 60
142	III	0 25>	0 50>	0 50	—	0 56	0 33	3 75	3 77
143	III	0 22>	0 50>	0 50	—	0 75	0 38	2 88	0 93
140	I	0 10	0 29	0 31	—	0 17	0 15	0 14	0 50
144	I	0 10	0 38	0 39	—	0 50	0 43	0 35	1 20

In three of the experiments the decerebration was definitely through the pons, in another (No. 133) it was just at the anterior border and in the remainder it was at other levels One hour after decerebration a 30 per cent solution of glucose was injected in several places under the skin It can be seen that the muscle glycogen scarcely changed in amount in those animals in which decerebration was performed elsewhere than through the pons, but became definitely reduced when it was at this level The sugar injection therefore failed to prevent the loss of glycogen from the muscles which usually follows pontine decerebration There is nothing noteworthy concerning the behaviour of the liver glycogen The only information which the results give us is that the stimulus causing disappearance of glycogen from the muscles after pontine decerebration is not influenced by the tendency for the glycogen to become built up again, such as occurs when this has been reduced by muscular activity It is usually believed that an excess of glucose in the blood stimulates the internal secretion of insulin and it is known that, under these conditions, glycogen is formed in the muscles That this did not occur in our experiments indicates that the stimulus to its breakdown was overpowering.

The increase in the percentage of sugar in the blood may depend merely on a readjustment of level without there being any measurable change either in the production or the loss of glucose It is known, for example, that a readjustment of blood sugar to a lower level may occur in richly-fed rabbits when insulin is injected, without any significant change either in respiratory metabolism or in glycogen formation (Chaikoff and Macleod, 1927) and that a marked and sustained hyperglycæmia may be induced by repeated injections of adrenaline into rabbits having very little, if any, measurable glycogen in the liver and muscles (Markowitz, 1924) We are, therefore, inclined to regard piqûre hyperglycæmia as being due not so much to an increased production or a decreased utilisation of glucose as to a breakdown of the mechanism by which these two processes are co-ordinated In the basal state, the blood sugar remains at a very constant level and it is only when there are very large demands for carbohydrate by the muscles, as after sustained severe exercise, that it shows a temporary fall This constancy must depend on a fine co-ordination between the production and utilisation of glucose in the organism, and it is this which may be interfered with by piqûre Whether this co-ordination is effected through the mediation of hormones, remains to be investigated. It is possible, for example, that the nerve control acts by causing an increased secretion of adrenaline when the blood sugar tends to fall, or of insulin when it tends to rise, but there is as yet no unequivocal evidence in support of such an hypothesis It is with these questions that we will concern ourselves in the third part of this report

Summary

(1) Decerebration at the pons in rabbits, besides causing hyperglycæmia has the following effects (*a*) increases the percentage of lactic acid in the blood, (*b*) decreases the percentage of glycogen in the liver when this is above about 0 7 to start with, but usually increases it when below this level, (*c*) decreases the percentage of glycogen in the leg muscles

(2) These changes are usually insignificant when decerebration is performed at other levels than the pons When decerebration is at the medulla oblongata the muscle glycogen usually increases in percentage

(3) The degree of hyperglycæmia after decerebration bears no relationship to the initial percentages of glycogen in the liver and the muscles

(4) The total amount of sugar which can be calculated as arising from the changes in the percentages of glycogen in the liver and the muscles during the course of a decerebration experiment in animals with low initial percentages of glycogen falls far short of that necessary to induce, by intravenous injection of glucose into a normal rabbit, a degree of hyperglycæmia comparable to that actually observed It is concluded that a process of gluconeogenesis must be responsible for most of the extra sugar which appears in the blood after pontine decerebration

In animals with high initial percentages of glycogen in the liver and the muscles, most of the extra sugar may come from this source

(5) The percentage of non-protein nitrogen of the blood was not found to be significantly increased after pontine decerebration

(6) The respiratory quotient is not affected by decerebration at the pons and the consumption of oxygen may either increase or decrease somewhat In one experiment the increase in oxygen consumption was pronounced

(7) Injection of glucose solution into animals decerebrated at the pons has no effect on the fall in the percentage of glycogen in the leg muscles, but may retard the decline in that of the liver, or cause it to increase

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Studies in the Nervous Control of Carbohydrate Metabolism
III —*The Nature of the Mechanism of the Nerve Control*

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The two preceding sections have been devoted to a study of the centre which controls the level of sugar in the blood Its location in the brain and the changes in carbohydrate metabolism resulting from its stimulation have been examined. The present section will be devoted to consideration of the results of certain experiments designed to throw light on the mechanism which is involved in this nerve control

The problems to be considered are (1) the nerve pathway through which the centre discharges its impulses, and (2) the particular mechanism upon which these impulses primarily act Investigation of the first of these would be greatly simplified if a definite answer could be given to the second, but, as we have seen, this is not possible, since functions of varying types located in different organs become involved Indeed, the widespread nature of the disturbances set up by stimulation of the centre suggests the possibility that they must be due to the action of some hormone, or hormones, whose secretion into the blood is regulated by the nerve stimulus If this be so the problem narrows itself down to finding the nerve pathway passing from the centre to the glands secreting the hormone Two glands, the adrenal and the isles of Langerhans, have to be considered, and there is much evidence that the hormone which each secretes, adrenaline and insulin respectively, is under nerve control But the nerves concerned apparently belong, in the one instance to the sympathetic (adrenal) and in the other, to the parasympathetic system (isles of Langerhans), so that the impulses resulting from piqûre might act through either pathway , through the spinal cord and splanchnic nerves to the adrenal gland so as to stimulate an increased secretion of adrenaline or through the vagus so as to inhibit the secretion of insulin In its rapidity of onset the hyperglycæmia which follows piqûre resembles much more closely that which follows the injection of adrenaline than that supervening upon pancreatectomy, so that most workers who have supported the hypothesis that the nerve control acts through hormones have paid attention only to the adrenal gland Our attention in the present paper will first of all be given to the effects of pontine decerebration on adrenalectomised animals

Effect of Adrenalectomy

Adrenalectomy was performed in two stages, the gland on the right side being removed a week or two before that on the left side Some time after the second operation, the animal was decerebrated at the pons There was considerable mortality, especially after the second operation, and only five animals were suitable for the decerebration experiments After adrenalectomy several were offered food rich in carbohydrates, but it is improbable that much was taken, unless in the animal of experiment 185 to which extract of adrenal cortex* was also given daily Two of the animals did not survive decerebration for more than 2 hours The results are given in Table I

In all instances, unless otherwise noted, no traces of adrenal tissue could be detected on post-mortem examination and the decerebration was definitely through the pons The blood sugar percentage in the first four experiments was practically unaffected 2 hours after decerebration (No 108 being discounted since the animal was dead when the blood was removed) Its initial value was low in one of the animals, being at the normal level in the others The blood lactic acid in three experiments showed a tendency to increase, but it is likely that this was merely due to failing circulation In the fifth experiment (No 185) decerebration was followed by a decided increase in blood sugar This animal had been kept for 2 weeks after the second operation during which time, perhaps because of daily injections of cortical extract, it took its food readily

It will be observed that the glycogen of the liver diminished in all the experiments, the loss being considerable in those in which fair percentages were present initially In one of the experiments (No 101), the glycogen of the muscles did not diminish , in two others the diminution was relatively small (Nos 108 and 170), but it was pronounced in the last experiment, in which there were large stores of this material to start with

These results must be collated with those of previous investigators, and since the latter have already been collected together and fully discussed by Stewart and Rogoff (1918), we need only refer to them briefly here Several (Mayer, 1906 , Kahn and Starkenstein, 1911 , Biberfeld, 1913) may be dismissed at

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* We have to thank Dr. R. A. Cleghorn for the extract, the activity of which he had determined on adrenalectomised cats

Table I—Effect of Pontine Decerebration on Adrenalectomised Rabbits

No of experiment	Blood sugar (S) and lactic acid (A)						Glycogen of muscles (M) liver (L)		Remarks
	Normal	Immediately after decerebration	1 hour	2 hours	3 hours	4 hours	Before	After	
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
101	S 0 100	0 100	0 109	0 129	0 121	0 113	M 0 394	0 402	Adrenalectomy 10 and 3 days previously A minute fragment of right adrenal left
	A 0 055	0 041	0 035	0 043	0 053	0 103	L 2 020	0 147	
106	S 0 049	0 045	0 036	0 040	—	—	M 0 108	Trace	Adrenalectomy 10 and 3 days previously Circulation very poor during second hour after decerebration
	A —	0 033	0 050	0 062	—	—	L 0 028	Trace	
108	S 0 114	0 097	0 102	0 184*	—	—	M 0 232	0 156	Adrenalectomy 7 and 1 days previously * Heart blood, animal dead
	A 0 078	0 050	0 058	0 150*	—	—	L 1 790	0 282	
170	S 0 092	0 094	0 092	0 097	0 097	—	M 0 382	0 322	Adrenalectomy 3 months and 4 days previously Cortex extract injected after second operation. Food withdrawn day before decerebration.
185	S 0 104	0 118	0 128	0 144	0 174	0 202	M 0 043	0 405	Adrenalectomy 21 and 14 days previously Cortex extract injected daily after second operation. Animal ate wheat and sugar up to time of decerebration experiment
							L 4 900	0 610	

once, since no determinations of blood sugar were made, the urine only being tested for the presence of sugar The great diminution in the excretion of urine which follows the operation of double adrenalectomy would in itself account for the absence of glycosuria in many of these experiments Freund and Marchand (1914) found a decided rise in the percentage of blood sugar in seven out of fourteen rabbits in which piqûre was performed by Eckhard's method after double adrenalectomy, which in most of the experiments was performed 4-6 hours previously Stewart and Rogoff criticise these experiments on the score that the effects of the anæsthetic used during the adrenalectomy operation may not have passed off Jarisch (1914) concluded, as a result of experiments in which the adrenal gland was denervated on the left side and removed on the right (five experiments), that piqûre, performed by Eckhard's method, had no effect on the blood sugar level The results are indecisive since only one sample of blood was examined for its sugar content after piqûre, which was made under ether anæsthesia In Stewart and Rogoff's own experiments piqûre was performed by Eckhard's method under local anæsthesia They found that hyperglycæmia did not necessarily follow piqûre in normal animals, even when a fair amount of glycogen was present in the liver It was pronounced in two rabbits adrenalectomised in two stages, but failed to develop in three others Their general conclusion is that piqûre may cause decided hyperglycæmia in rabbits which have survived the removal of both adrenals Evans, Tsai and Young (1931) have clearly shown that removal of the adrenals has the effect of greatly diminishing the hyperglycæmia and in accelerating the rate of restoration of glycogen in the liver following decerebration in cats

Considering the previous observations along with our own, it seems clear that although hyperglycæmia may result from piqûre in adrenalectomised rabbits, it often fails to do so When made through the floor of the ventricle some of the failures may have been due to non-involvement of the pons, but this cannot explain them in the present experiments in which decerebration was found on post-mortem examination to be definitely at this level Two other possible causes for the absence of hyperglycæmic effect have to be considered, (1) lack of glycogen in the liver and muscles, and (2) interference with the conductivity in the efferent nerve pathway concerned in regulating the blood sugar level Since we have already shown that pontine decerebration causes hyperglycæmia when only traces of glycogen are present in the liver, and since, in two of the present experiments (Nos 101 and 108) decerebration failed to cause hyperglycæmia although the losses in liver glycogen were decided, we do not consider that the presence or absence of this material is the main factor upon which hyperglycæmia depends It will be observed, moreover, that the decreases in muscle glycogen were very much less pronounced following decerebration in the adrenalectomised animals than in normal ones (*cf* Table II of second paper) When the glycogen stores in both liver and muscles were large, as in the last experiment of the table, the hyperglycæmic effect of decerebration was present and much glycogenolysis occurred in both liver and muscles, so that it is possible that the sugar thus derived along with that which was probably being absorbed from the alimentary canal, accounts for the result If this be the correct interpretation, then we must conclude that the diabetogenic impulses set up by decerebration may, after adrenalectomy, act on the glycogen of the liver and, to a less extent, on that of the muscles By removal of the adrenals, the body appears to be deprived of something, probably adrenaline, which is necessary for maintaining at its normal pitch the excitability of the nerve mechanism by which the blood sugar level can be raised This same conclusion was arrived at by Macleod and Pearce (1912), as a result of experiments concerning the influence of adrenalectomy on the hyperglycæmic effects of stimulation of the splanchnic and hepatic nerves They considered that the presence of the adrenal is necessary to maintain the functional integrity of the peripheral endings of the glycogenolytic nerve fibres The results of the present investigation would seem to show that this applies also to the muscle glycogen

The Effect of Vagotomy and Atropine

It has already been suggested that there are two types of mechanism by which excess of sugar is discharged from the liver into the systemic blood following decerebration When, as in an animal richly fed with carbohydrate, large amounts of glycogen are being stored away in the liver, much of the extra sugar comes from this source and the nerve impulses which set up the glycogenolysis act independently of the presence of the adrenal glands When, as in a previously fasted animal, the glycogen disappearing from the liver represents only a small proportion of the extra sugar produced by decerebration, the nerve impulses cannot act unless these glands are present This gluconeogenic glycogen, as we may call it, represents only what remains over in an ever-changing process in which it is being built up out of non-carbohydrate material and broken down into glucose, and this process may be under a different type of nerve control from that of the stored glycogen Since, for various reasons, it has been assumed that the stored glycogen in the liver is under sympathetic nerve control, we were led to examine whether decerebration hyperglycæmia would occur after blocking the parasympathetic nerve pathway, by section of the vagi and administration of atropine It was found that under these circumstances decerebration had very little, if any, effect on the blood sugar level, provided there was not a very large percentage of glycogen in the liver The procedure followed in performing decerebration, under temporary amytal anæsthesia, was exactly as previously described, and in all instances post-mortem examination showed that decerebration was through the pons The results are shown in Table II

After section of the vagi alone (Nos 165 and 167) decerebration did not cause the usual increase in blood sugar, and when atropine was also injected (Nos 168–175) this absence of hyperglycæmic effect was, as a rule, still more striking Only in one experiment (No 172) did the blood sugar rise above 0 200 per cent. This was the only experiment of this series in which the liver glycogen was determined and, finding it to be unusually high for a fasted animal, the question arose whether the presence or absence of hyperglycæmia might depend on the magnitude of the glycogen stores In the next series of experiments (Nos 176–180 inclusive), therefore, some of the animals were specially fed with readily assimilable carbohydrate food mixture (ground maize and sugar) up to the time of the experiment while others were fasted, and the glycogen in liver and muscles was determined in all of them The results show that when there are large stores of glycogen (Nos 177 and 178), atropine and vagotomy do not prevent the development of the hyperglycæmia or protect the glycogen of the liver against rapid disappearance When the glycogen stores are small they may or may not become reduced (Nos 179 and 180) It is possible that the breakdown of glycogen in the muscles is less marked than when no atropine is given (compare with Table I in Section II), but of this we are uncertain

Since, as we have already seen, decerebration usually fails to cause hyperglycæmia in animals kept continuously anæsthetised by amytal (p 127), the foregoing results with atropine suggested the possibility that the two drugs act in the same manner This is supported by the observations of Olmsted and Girragosswitz (1931) which show that a slight rise in blood sugar occurs after amytal in dogs fed on a diet rich in carbohydrate The effects of amytal in abolishing the inhibitory effect of the vagus on the heart beat (Lieb, 1929; Shafer, Underwood and Gaynor, 1930, Garry, 1930) and in stopping the peristaltic movements of the gastro-intestinal tract (Weiss, 1929) indicate a paralysis of parasympathetic nerve supply of these functions also, and the

Table II —Behaviour of Blood Sugar and Glycogen Percentages following Pontine Decerebration in Vagotomised and Atropinised Rabbits

No of experiment	Nutritive condition	Blood sugar normal	Blood sugar immediately after decerebration	B S at hourly intervals.				Glycogen.			
				1	2	3	4	Before	After		
		per cent.	per cent	per cent	per cent	per cent	per cent	per cent	per cent		
165	Fast	0 118	0 141	0 132	0 136	0 226	0 226	—	—	Vagi cut	No atropine
167	,,	0 129	0 122	0 125	0 156	—	—	—	—	,,	,,
168	,,	0 108	0 142	0 142	0 140	0 164	0 191	—	—	Vagi cut repeatedly	Atropine injected
169	,,	—	0 121	0 106	0 163	—	—	—	—	,,	,,
171	Fed	0 109	0 097	0 104	0 115	0 146	0 149	—	—	,,	,,
172	Fast	0 098	0 094	0 130	0 181	0 216	0 328*	—	L 3 79	,,	,,
173	,,	0 096	0 086	0 096	0 086	0 113	0 186†	—	—	,,	,,
174	Fed	0 111	0 127	0 204	0 208	0 224	—	—	—	,,	,,
176	Fast	0 105	0 139	0 133	0 133	0 133	0 180	L 2 530 M 0 406	L 0 788 M 0 389	,,	,,
177	,,	0 105	0 212	0 255	0 294	0 411>	0 486	L 12 200 M 0 503	L 7 170 M 0 396	,,	,,
178	Fed sugar	0 094	0 158	0 188	0 390>	0 466	—	L 16 400 M 1 445	L 9 700 M 1 285	,,	,,
179	Fast	0 096	0 138	0 132	0 098	0 079	—	L 0 445 M 0 482	L 0 198 M 0 343	,,	,,
180	,,	0 098	0 098	0 091	0 095	0 089	0 089	L 0 188 M 0 074	L 0 161 M 0 063		

* Breathing poor † Heart blood

question arises whether it acts similarly on the nerve supply of glands The answer would appear to be in the affirmative Thus, in two experiments, which we performed along with Dr Magee, the chorda tympani nerve to the submaxillary gland of the etherised dog was stimulated, before and after injecting amytal intravenously, and it was found that the resulting secretion of saliva was very greatly diminished for some time after the injections We conclude, therefore, that both atropine and amytal institute a block in the parasympathetic nerve supply of the gluconeogenic function of the liver The centre for this supply must be situated in the pons and become stimulated by decerebration and piqûre at this level Since adrenalectomy also prevents the development of hyperglycæmia following decerebration, the presence of adrenaline in the blood would appear to be essential for maintaining the functional integrity of the parasympathetic nerve control of gluconeogenesis We do not know whether the parasympathetic nerve control of other glands, such as the submaxillary, is affected by adrenalectomy

There remains to consider the nature of the nerve control of the mechanism of storage of liver glycogen As we have seen, when stored glycogen is abundant neither atropine nor adrenalectomy (nor probably amytal) prevents decerebration hyperglycæmia, and this raises the question whether the nerve pathway involved in its production may be the sympathetic To answer it, we have studied the effect of decerebration after section of the hepatic nerves and after injecting ergotamine Although the results have not been satisfactory, they merit some reference here Ergotamine was injected in experiments 91, 93 and 95 (Table V of Section I) The hyperglycæmia was already well established when the injections were made and no effect was observed in two of the experiments, although the hyperglycæmia became less marked in the third The use of this drug was then abandoned, partly because of the great difficulty in collecting blood from the ear veins after its use and partly because the pulse became very feeble, indicating failing circulation We have more recently repeated these experiments with the difference that the ergotamine was injected immediately after decerebration, the rabbits having been either specially fed with maize and sugar, or fasted for 48 hours The results are shown in Table III

It is clear that ergotamine does not prevent the hyperglycæmia or diminish the rate of disappearance of glycogen from the liver following decerebration in rabbits with large stores of glycogen, although it may slightly retard the rate of disappearance of glycogen from the muscles. On the other hand, it prevents the development of hyperglycæmia in fasted rabbits with little glycogen.

Table III —The Effect of Ergotamine* on Decerebration Hyperglycæmia

No of experiment	Nutrition condition.	Blood sugar normal	Blood sugar immediately after decerebration	B S. at hourly intervals after decerebration						
				1 hour	2 hours	3 hours	4 hours	Before	After	
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
181	Fast	0 090	—	0 089	0 102	0 101	0 108	L 0 97 M 0 67	0 45 0 39	—
182	,,	0 080	0 092	0 102	0 106	0 078	—	L 0 18 M 0 18	0 10 0 07	—
183	Sugar fed	0 098	0 104	0 131	0 250	—	—	L 10 50 M 1 12	6 65 0 89	Vagi also cut
184	,,	0 103	0 142	0 127	0 239*	0 485	—	L 7 20 M 1 13	2 90 0 72	,, Ergotamine repeated
185	,,	0 102	0 152	0 090	0 165	0 172	—	L 6 90 M 0 70	2 67 0 65	Vagi cut and atropine also injected.

* 0 5 mg ergotamine was injected just before decerebration Owing to constriction of the ear vessels, the blood samples had sometimes to be taken from the carotid

These results are, therefore, like those obtained after atropine, and when both drugs are given together in a glycogen-rich rabbit (as in No. 186) the blockage of the nerve pathway would appear to be more complete than with either separately.

The hepatic plexus of nerves was cut in four experiments (Table IV) This was done by carefully separating the portal vein from the adjoining tissues, including the hepatic arteries and bile duct, and cutting them between ligatures This is a difficult operation in the rabbit and we are uncertain whether we invariably succeeded in cutting every branch of the plexus The isolated portal vein was usually sponged with 10 per cent carbolic acid solution to destroy any nerve fibres on its wall Experiments 110 and 121 served as controls, the nerves being cut without performing decerebration In No 112 decerebration was performed immediately after the nerves had been cut. In Nos 115 and 123 the decerebration was performed in 1 hour and in 2 hours respectively after cutting the nerves

Table IV —The Effect of Section of the Hepatic Nerves on Decerebration Hyperglycæmia

No of experiment	Blood sugar normal	B S at hourly intervals					Remarks
		0	1	2	3	4	
110	0 118	0 127	0 136	0 159	—	0 166	Controls
121	0 108	0 160	0 254	0 347	—	—	
112	0 116	0 228	0 228	0 218	0 232	0 203	—
115	0 099	0 183	0 261*	0 313	0 325	—	*Decerebration after this sample
123	0 126	0 185	0 216	0 266*	0 347	--	

Since the operation in itself usually caused hyperglycæmia, probably because of disturbances in the portal circulation, the investigations were not pursued further The results, so far as they go, indicate that decerebration hyperglycæmia still occurs after section of the hepatic nerves We have not as yet attempted the obvious experiment of performing decerebration after section of the splanchnic nerves In order to avoid disturbance of the abdominal viscera at the time of the decerebration experiment, it would be necessary to perform the splanchnotomy several days previously and this we have not as yet found time to do

It is interesting to compare these results with those obtained by others who have studied the effects of atropine and ergotamine on carbohydrate metabolism. McGuigan (1916) states that atropine has no influence on the hyperglycæmia due to ether or to stimulation of the cœliac plexus. Allard (1926) observed on man that 1 mg. atropine entirely prevented the hyperglycæmia which occurs when glucose is given by the mouth, and suggests that this is because it inhibits hepatic glycogenolysis. Pollak (1928), using rabbits, found that the effect of atropine or of ergotamine on alimentary hyperglycæmia, although irregular, was often to suppress it and this result was always obtained when the two drugs were given together. The hyperglycæmia caused by subcutaneous or intravenous injection of sugar, on the other hand, was not influenced by the drugs, either separately or combined. Since the inhibition of alimentary hyperglycæmia occurred in fasted animals, in which it is assumed that there is no glycogen in the liver, Pollak considers that it cannot be due to suppression of glycogenolysis, but is probably dependent on an effect on the rate at which the liver removes sugar from the portal blood. This interpretation receives support in the fact that the above drugs also suppress the alimentary hyperglycæmia following ingestion of fructose or galactose.

The fact that atropine and ergotamine inhibit alimentary hyperglycæmia and have a like action on decerebration hyperglycæmia in fasted rabbits may, we think, be due to their blocking the nerve pathway through which either decerebration or alimentary absorption of sugar ordinarily produces it. This explanation presupposes that gluconeogenesis is, in part at least, under nerve control and also that the centre concerned is reflexly stimulated during the intestinal absorption of sugar. When sugar is being absorbed it would appear to stimulate sugar secretion by the liver at the same time that most of it is being deposited as glycogen. Evans, Tsai and Young (1931) also consider this possibility but from a somewhat different point of view. Reverting to Claude Bernard's comments on the same subject, they conclude that when glucose is administered, especially by the portal vein, the laying down of glycogen which occurs "may be due to retention of new-formed glycogen, which would otherwise have been converted into blood sugar, just as much as to a direct deposition of glucose from the blood stream as glycogen."

Space will not permit of a full discussion of all the implications of the above hypothesis. We may, however, refer to the effects of sub-convulsive doses of insulin on rats and of pancreatectomy on dogs. After fasting, the immediate effect of insulin is to cause a decrease in liver glycogen and during the ingestion of carbohydrate to retard its accumulation (Barbour, Chaikoff, Macleod and Orr, 1927). After pancreatectomy glycogen almost disappears from the liver and does not accumulate when sugar is fed. All this fits the hypothesis, originally propounded by Laufberger, that one of the principal effects of insulin is to inhibit the rate of gluconeogenesis in the liver In its absence, as after pancreatectomy, gluconeogenesis becomes maximal and when it is so, glycogen is apparently not so readily deposited from absorbed glucose, give insulin, gluconeogenesis stops and the process of glycogen formation sets in again When insulin is present in excess, as after its injection in sub-convulsive doses, it inhibits gluconeogenesis so that the demands of the organism for carbohydrate have to be met, either by drafting whatever glycogen remains over in the liver as a product of the preceding gluconeogenesis (in fasted animals) or by using some of the absorbed sugar (in fed animals). Insulin therefore acts on gluconeogenesis in the opposite sense from the nerve stimulus which is set up in exaggerated form by decerebration and in lesser degree by absorption of sugar from the alimentary canal, and it is significant that insulin prevents the rise of blood sugar following piqûre (Banting, Best, Collip, Macleod and Noble, 1922) and likewise reduces or may prevent the hyperglycæmia following decerebration, as we have shown These considerations might at first sight lead one to infer that insulin really acts like atropine, in that it paralyses the gluconeogenic nerve impulses, but its action must be more directly on the gluconeogenic process itself, otherwise atropine or vagotomy would reduce the hyperglycæmia in pancreatic diabetes which, as far as we know, is not the case We do not mean to imply that insulin does not also influence the process of glycogen formation in the muscles, but this is not within our present discussion

It may seem difficult to harmonise these conclusions with the apparently established fact that the vagus contains insulin-secretory fibres to the pancreas, but it must be remembered that the fall in the blood sugar percentage which follows stimulation of this nerve has usually been demonstrated either on amytalised animals or on those from which the adrenal glands have been removed or the hepatic nerves cut We have seen that decerebration does not cause hyperglycæmia under such conditions, at least when only moderate amounts of glycogen are present in the liver

To sum up, decerebration hyperglycæmia does not occur in rabbits having small amounts of glycogen in the liver after the following (1) atropine, (2) ergotamine, (3) amytal, (4) insulin, (5) adrenalectomy Under the same conditions, alimentary hyperglycæmia is also suppressed by atropine, ergotamine and insulin Atropine, ergotamine and amytal probably act by blocking the nerve pathway through which the decerebration stimulus acts on the gluconeogenic process, insulin by directly inhibiting this process and adrenaline, when in the small concentration present in normal blood, by maintaining the functional conductivity of the nervous pathway and, when present in excess, by stimulating the gluconeogenic process directly We do not yet possess sufficient information to indicate what relationship the process of glycogen deposition bears to that of gluconeogenesis When the latter is suppressed, which is presumably the case when glycogen is being rapidly formed, as a result of the absorption of carbohydrates, decerebration hyperglycæmia does not occur after atropine, ergotamine or amytal, but probably does so after insulin This would lead us to infer that the impulses set up by decerebration in animals in which the liver is overloaded with glycogen act through some other nerve pathway—it may be indirectly through some ductless gland—than that controlling gluconeogenesis When we found that the latter impulses were blocked by vagotomy and by atropine, the possibility suggested itself that they might be transmitted exclusively by the parasympathetic, reserving for the sympathetic the glycogenolytic impulses acting on stored glycogen The results with ergotamine and following adrenalectomy, when very large amounts of glycogen are present in the liver, do not support such a view It is, of course, possible that under these conditions vascular disturbance is in itself sufficient to set up glycogenolysis and that decerebration causes this disturbance Several investigators have noted that the liver is very much congested after piqûre, and it is well known that decerebration is frequently followed by hyperpyrexia The increased flow of blood at a temperature above the normal may have the effect of causing the stored glycogen of the liver to break down rapidly into glucose

Summary

(1) In rabbits in which there are small amounts of glycogen in the liver and muscles decerebration at the pons does not cause hyperglycæmia under the following conditions (*a*) double adrenalectomy, (*b*) atropine and section of both vagus nerves, (*c*) ergotamine and section of both vagus nerves, (*d*) amytal.

(2) In rabbits in which there are larger amounts of glycogen in the liver and muscles, as a result of recent feeding with carbohydrates, decerebration at the pons does cause hyperglycæmia, under the above conditions, but it is usually not so pronounced

(3) The influence of section of the hepatic nerves on decerebration hyperglycæmia is uncertain, since this operation in itself is usually followed in the rabbit by an increase in the percentage of blood sugar.

It is considered that when there is little glycogen in the liver decerebration hyperglycæmia is due to stimulation of the gluconeogenic process in the liver by way of the parasympathetic nerves

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The Differential Action of X-Rays in Relation to Tissue Growth and Vitality Part VI—Tumour Tissues.*

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[Plate 5]

This paper on tumour tissues is an extension of previous work on the reaction of the allantoic membrane to homogeneous X-radiation (1930, 1931), and the relation of such results to mixed radiation and to tissues generally

It is also an extension of work on the treatment of tumours with mixed radiation, of which Russ (1921) was a pioneer

The present work corresponds in particular with the *in vivo* experiments of Russ, but the various results appear more definite or clear cut, and it is hoped that homogeneous radiation will furnish an instrument of greater precision

Particular emphasis has been laid on the subject of immunity mainly from the inspiration of Lumsden's serological work (1925)

Physical Arrangements.

Previous work (1931) has indicated that homogeneous radiation of a frequency range not exceeding 20 per cent. might produce effects with a small fraction of the dose required for mixed radiation, and an intermediate dose might be required for intermediate types of radiation

Three sources of energy were used, a "lathe bed" spectrometer, and a multiple spectrometer, and a source of homogeneous K radiations (Moppett 1930), representing graduated steps connecting mixed and homogeneous radiation

With the lathe spectrometer A the frequency range was approximately 8 per cent, the mean wave-length 0 53 Å U, and the exposure 3 hours at 15 ma, similar to that used for mouse skin, and 15 times the threshold hypertrophic dose for allantoic membrane, or approximately 9 R.

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* This work was carried out under the control of the Cancer Research Committee of the University of Sydney with the aid of the Cancer Research and Treatment Fund.

With the multiple spectrometer B the frequency range was approximately 20 per cent, the mean wave-length 0 53 Å U., the exposure 2 hours, and the incident energy 50 R by a rough photographic comparison

Homogeneous K radiations appeared to furnish the only means of treating a large mass of tissue on the above lines It was thought that a wave-length of 0 5 Å U. (approximately) would be unsuitable as the energy and homogeneity is considerably reduced even by 1 cm of tissue The next peak of effect in the allantoic membrane 0 3 (1930) presented difficulties in the choice of target element and uranium was adopted because the K lines (0 10842–0 13095 Å U) cover the region of the first peak

The radiation emerged in the direction of the cathode rays, an arrangement which brings the peak of the energy curve nearest to the quantum limit and filtration was provided by the target substance together with 1 mm of copper and a water space 1 cm wide to remove residual soft radiation

A spectrogram is reproduced in fig 1, Plate 5, with wave-length scale and a density tracing taken with a Moll microphotometer

It is not possible to treat the question of homogeneity in the previous manner since there are a series of intense lines (two only can be distinguished owing to scattering) with an unavoidable fringe of hard and soft radiation

If one chooses an arbitrary frequency band of intensity δ to include most of the lines, and ϵ represents the integrated intensity of all other frequencies, the ratio $\delta/\delta + \epsilon$ would express the homogeneity in accord with previous work with crystal diffraction (1931)

Various doses were given in terms of time of exposure and investigation with a standardised Solomon ionometer showed that 1 R equals 1 milliamp minute within the limit of experimental error

Biological Material

The tumour tissue was a strain of a mouse sarcoma (S37 of the Imperial Cancer Research Fund), kindly lent to me by Dr Horning Composed originally of round cells, there has been a tendency to polymorphism with later passages, fig 2, Plate 5 With weekly inoculation the tumour was raised to a high virulence which has remained constant for over 2 years. White mice were used for convenience in numbering, and of a series of 537 there have been only rare immune individuals (failure to take) and four cases of spontaneous retrogression giving a probability of 1/134

The tumour was implanted in two places under the skin of the back in the cervical and lumbar regions and the anterior growth only was irradiated.

Usually six animals were inoculated of which 1 served to perpetuate the strain, 2 were controls, and 3 were available for experiment The irradiated animal (and some of the controls) were fixed to a wooden frame by two stitches so that the tumour and an inevitable fringe of normal tissues were irradiated through a port of 1 sq. cm

Irradiation took place 1 week after inoculation, when the tumours were on an average 0 3 cm in diameter and observations were made at weekly intervals thereafter, death usually taking place about the end of the fourth week

Experimental Results

The results with the lathe bed spectrometer are recorded in Table I, A. Twelve experiments were carried out together with 12 controls, and, except in the case of one advanced tumour, the action of the X-radiation could be readily recognised by comparison with the untreated tumour and also the tumours in the control animal

A few days after irradiation, a dark red area of skin was observed which suggested a subjacent hæmorrhage Later this region became distinctly hard to the touch and in contrast with the continued growth of the peripheral regions it presented a depression Microscopically such a tumour showed a mass of deeply staining debris where the tumour permeated the skin corresponding to the above-mentioned area Deeper in the tumour the loss of nuclear stain showed the presences of large areas of degeneration, but such appearances are not uncommon in untreated tumours and, on the whole, the naked eye examinations were more convincing When observation was continued for a fortnight, it was observed in nine cases that the peripheral parts of the irradiated tumour showed more rapid growth than the corresponding tumour which had not been irradiated The three remaining animals died before observation was complete, but in all the above instances what may be described as unsuccessful treatment lead to stimulation

Observation of all four tumours in the irradiated and control mice was possible up to the third week in seven instances. In five such cases not only was the irradiated tumour stimulated, but the non-irradiated tumour began to grow more rapidly than those of the control animal In two cases the rate of growth was about equal, but from the ratio 5/7 one may state tentatively that the unsuccessful treatment caused not only a local stimulation, but possibly as a secondary effect a " systemic stimulation " or condition of negative immunity.

Series B, Table I, comprised 20 experiments of which 5 were lost in an epidemic Of the remainder 6 were controls and 5 showed stimulation first of

Table I

Source of rays.	Experiments		1 week after irradiation.		2 weeks.		3 weeks	
A. Lathe spectrometer Dose approx 9 R	Controls	12	Progressive	12	Progressive	12	Progressive	12
	Irradiated	12	Umbilication Doubtful	11 1	Stim irrad Dead	9 3	Stim. both Stim irrad. Dead	5 2 5
B Multiple spectrometer Dose approx 50 R	Controls	6	Progressive	6	Progressive	6	Progressive	6
	Irradiated	14	Umbilication Dead	9 5	Stim irrad Dead	9 5	Stim both Cure and immune Dead	5 4 5
C Uranium K lines 1 mil amp min = 1 R, approx 40 ma.m	Controls Irradiated	2 4	A preliminary induration and appearance of a scar is usually observed		Stim irrad Dead	3 1	Progressive Stim both Stim. irrad Dead	2 1 2 1
C Uranium K lines 1 mil amp min = 1 R, approx 80 ma.m	Controls Irradiated	8 12	A preliminary induration and appearance of a scar is usually observed		Stim irrad	12	Progressive Stim. both	8 12
C Uranium K lines 1 mil amp min = 1 R, approx 100 ma m	Controls Irradiated	4 6	A preliminary induration and appearance of a scar is usually observed		Growth unaffected		Progressive Progressive	4 6
C Uranium K lines 1 mil, amp min = 1 R, approx 120 ma m	Controls	19					Progressive	19
	Irradiated	30	A preliminary induration and appearance of a scar is usually observed		Ulceration of both tumours usual, death apparently results from toxæmia rather than continued growth		Retard both Cure and immune Cure irrad Cure non-irrad No effect Early death	18 4 4 1 1 2
C Uranium K lines 1 mil amp. min = 1 R, approx 160 ma m	Controls Irradiated	6 9	Rapid ulceration		Ulceration both tumours		Progressive Continued growth	6 9
C_1 Uranium K lines 120 ma.m plus prior dose soft mixed rays, 5 R	Controls Irradiated	2 3	No ulceration but growth				Progressive Progressive	2 3

Totals Controls 59, irradiated 90 Probability of spontaneous retrogression 1/134 (Dead controls not recorded.)

the irradiated tumour and later of the non-irradiated tumour as described above In 4 cases the tumours exhibited the early reactions described and later became hardened and towards the middle of the third week both the irradiated and non-irradiated tumour broke down quite suddenly, leaving an open abscess in which no further tumour growth was observed during the life of the animal

From these results it was assumed that one had just attained the threshold dose for producing the Lumsden's "antigen stage," there being an approximately equal number of experiments, showing stimulation and negative immunity and cure and immunity

The K radiations provided great elasticity in dosage and the results will be dealt with in groups which are separated by horizontal lines in the Table I, C

A small dose (40 to 80 R) produced a stimulation comparable in every way with that of Series A and B

A slightly larger dose (100) appeared to lead to a balance of different factors and the ultimate growth was little affected When the dose is increased to 120 there is a very definite retarding action, and in 4 cases out of 30 both tumours disappeared The ratio of cures was low, but the tumour was very virulent, slightly more so than during the early experiments in Series B. The ulceration recorded in the tables took place in both irradiated (cervical) and non-irradiated (lumbar) tumours, and was undoubtedly connected with the curative process (rather than casual infection) but several animals with retrogressing tumours died apparently of toxæmia before a cure could be recorded.

A section of retrogressing tumour shows a massive central degeneration merging into the open ulceration, but there is also a peripheral fibrous reaction and round-cell infiltration recalling the peripheral reaction observed by Lumsden

The non-irradiated retrogressing tumour shows similar changes with rather less central degeneration and an example is given in fig 3, Plate 5, which may be compared with the normal tumour, fig 2, Plate 5, invading a corresponding region of the subcutaneous tissues

The next series with a larger dose (160+) were of interest because the "overdose" showed no curative properties, indeed, there was a suspicion of stimulation although ulceration was profound in both tumours

Lumsden has shown the importance of retaining autolytic tumour products in producing a cure and in Series C_1, an attempt was made to protect the skin from the destructive action of the hard rays by means of a prior dose of soft mixed rays on the lines of previous work with the allantoic membrane (1931).

There was no ulceration, but, unfortunately, the tumour growth was unaffected, probably because the soft radiation penetrated sufficiently to interfere with the subsequent reaction of the tumour mass to hard radiation The possibility of further work on these lines was investigated by means of normal rat skin.

Antagonism of Rays in Rat Skin.

The skin covering the back of a white rat was immobilised beneath a wooden frame by means of four stitches A small " T "-shaped area was then exposed to unfiltered mixed rays from a Universal Coolidge tube running at 25 KV., and immediately after the whole back was exposed to homogeneous K rays (C) according to the doses given in Table II

Table II

Prior dose mixed rays 3 ma = 4 R approx	Dose K rays uranium 1 ma = 1 R approx	Result	
		Central " T "	Periphery
ma	ma		
3	120	—	—
3	300	Visible 3 days	E
3	300	Visible 2 weeks	E
5	240	Visible 2 weeks	E
5	240	Visible 5 days	E
5	300	Visible 4 days	E
6	300	—	Uniform E
8	270	Visible 2 days	E
8	300	—	Uniform E
8	300	Visible 7 days	E
19	150	Erythematous T only	—
19	240	—	Uniform E

Reliability of conclusion, 7/9

Complete epilation was not produced and the hair was removed with sodium sulphide a few days after irradiation to permit one to observe the early erythematous changes

In accordance with the table, in 7 cases out of 9 (excluding obvious errors of dose) there was a definite protective action shown by an absence of erythema over the T-shaped area although more energy was received The effect was visible for periods varying from 2 days to several weeks, but, unfortunately, the slight yellowish tinge of the unaltered skin prevented a successful photographic representation. At a late stage desquamation and regrowth of hair make the outlines somewhat indistinct, but a comparison with the outline of the mask showed a close correspondence with the thicker hair in the " protected " skin

Clinical Experiments.

In the absence of a supply of spontaneous animal tumours some experiments were made with the K radiations on clinical material The restrictions of a physical laboratory only permitted the treatment of five epitheliomata of the dorsum of the hand, according to the details given in Table III

Table III

Tumour	Treatment	*Result*	*Second biopsy*
1 Squamous epithelioma (dorsum, hand)	120 R, 180 R	Reduced 1/10 original volume	Fibrosis
2 Hard warty growth (dorsum hand, no biopsy)	100 R	Reduced 1/3 original volume	—
3 Squamous epithelioma (dorsum, hand)	40 R, 80 R, 180 R, 150 R, 180 R	Good response at first, finally became radio-resistant	Fibrosis
4. Squamous epithelioma (dorsum, hand)	120 R, 120 R, 180 R, 180 R, 150 R, 180 R, 210 R	Rapid response at first, but inaccessible portion continued to grow and finally became radio resistant	Moderate round cell infiltration
5 Squamous epithelioma (dorsum, hand)	180 R, 180 R, 180 R, 210 R	Rapid response to each treatment	Scar with a few nodules

In assessing the results it must be remembered that such growths are very radio-resistant and the dosage at any one sitting was restricted to about one-quarter of the estimated erythema dose (for usual radiotherapy practice), because of the difficulty of running a demountable tube on heavy load

Repeated doses were given, but only at intervals of about 1 month, since severe erythematous reactions were obtained on the surrounding skin. The practice proved bad because the tumours increased in radio-resistance.

In every case some result was obtained, particularly after the first few doses, and a check was made on initial and final biopsies in all but one patient.

Nos 1 and 3 exhibited initially typical squamous epitheliomata, whilst in the final examination there was a dense fibrosis, apparently with healthy malignant cell groups in parts and residual necrotic masses in other parts In No 4 the second biopsy showed every evidence of increased malignancy, possibly a case of stimulation and the stroma was looser with many round cells and capillary blood vessels.

No 5, the best clinical result, exhibited a scar with no trace of tumour cells, except in a few nodules where the epithelial islands were reduced to thin shells by excessive keratinisation. Here there was a moderate round-cell infiltration, but no fibrosis and the general picture suggested a cessation of active growth.

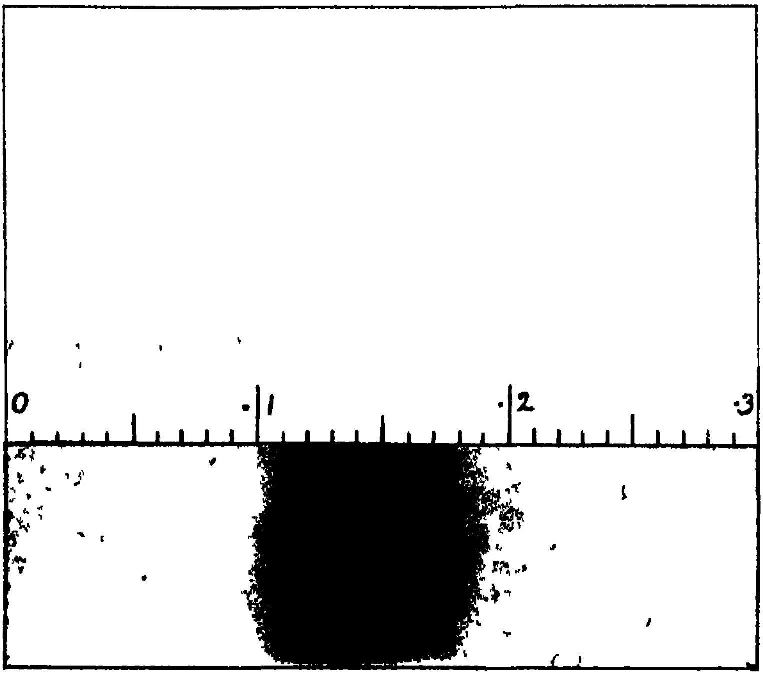

Fig 1

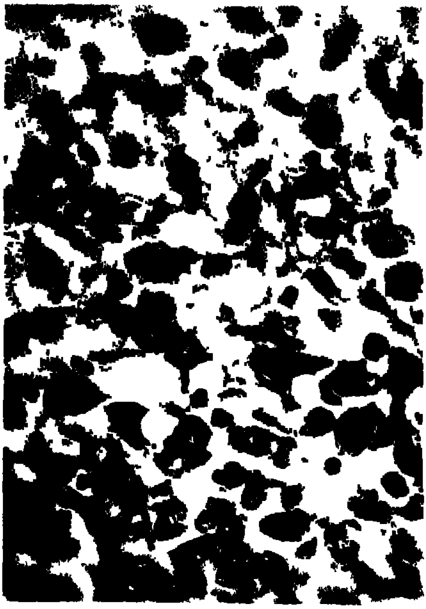

Fig 2

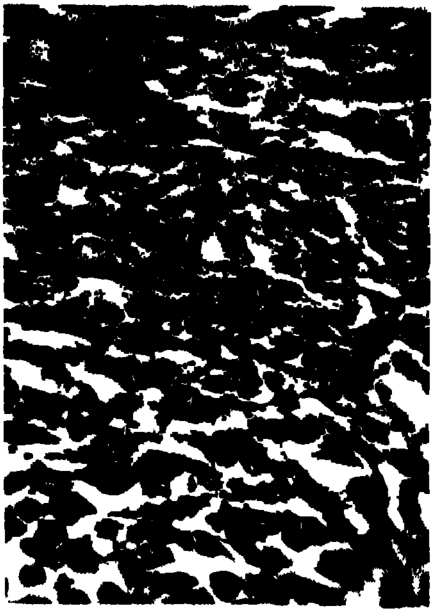

Fig 3

(*Facing p* 170)

Conclusions.

The changes produced by means of the lathe spectrometer A and the multiple spectrometer B serve to link up a tumour tissue with antagonism and selective action as a dual phenomenon in the manner already described for normal tissues

Viewed from the more practical standpoint of treatment, there are three well-marked stages, that of under dose and stimulation, that of retardation and cure, and that of overdose

Stimulation merely implies rapid growth relative to a control and the phenomena may well be a secondary stimulation to an inadequate injury (for which there is microscopic evidence) as opposed to the primary stimulation inferred in the case of the allantoic membrane (1930)

The systemic phenomena of positive or negative immunity have a counterpart in Lumsden's serological work and possibly in radiotherapy. The advisability of an adequate trial of K radiations is indicated apart from the obvious advantage of a constant wave-length in energy measurement

The demonstration of " prior action " in the rat similar to that of the allantoic membrane (1931) would be of great theoretical and practical importance, but in a complex tissue one must consider some other factor, such as vascular spasm, at least as a remote possibility

I wish to thank Drs Chapman and Sandes, Directors of Cancer Research and Treatment, and Dr Kenny for their interest in different aspects of this work

DESCRIPTION OF PLATES

PLATE 5

FIG. 1 —The separation of the uranium lines shows only in the density graph. The broad point lines in the photograph are due to tungsten spluttered from the cathode after long use

FIG 2 —Sarcoma cells (normal) invading subcutaneous tissues.

FIG. 3 —Margin of retrogressing tumour showing damaged sarcoma cells (below) and tissue reaction (above)

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The Relation between the Anterior Pituitary Body and the Gonads. Part IV—Induction of Ovulation during Pregnancy and its Effect on the Fœtuses.

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(Communicated by C Lovatt Evans, F R S.—Received December 4, 1931)

(From the Department of Physiology, University College, London)

I *Introduction*

There is considerable difference of opinion as to the precise effects produced by the administration of the ovary-stimulating hormones during pregnancy. Such discrepancy is not surprising when one considers that, according to the method of preparation and administration, extracts of anterior pituitary tissue call forth varying ovarian responses in test animals Further, considering the crudeness of many of the extracts used, there is always the possibility of direct action on the uterine musculature or of effects due to toxicity

Several workers have investigated the effect of anterior pituitary implants on the pregnant mouse and rat Engle and Mermod (1928) gave daily transplantations of fresh anterior lobe to a large number of mice and rats in early, middle and late pregnancy Implants in the middle of gestation caused reabsorption or expulsion of the fœtuses, but in latter stages of pregnancy the fœtuses often seemed to be unaffected and normal litters were obtained These authors consider, however, that the hormone in sufficiently large amounts would always cause abortion Engle and Mermod emphasise that ovulation only occurred when pregnancy had terminated, either after abortion or when the fœtuses were dead.

Zondek and Aschheim, on the other hand, found implantation of fresh anterior pituitary (0 05–0·1 gm) produced ovulation, while the fœtuses remained alive in the uterus , smaller amounts were without effect, but larger amounts caused abortion The difference between the results of Smith and Engle, and those of Zondek and Aschheim may be due to the fact that the latter only used one implant, whereas the former gave daily implants. Zondek and Aschheim observed that though they obtained ovulation, the œstrin produced was not enough to affect the vagina. Fels (1928) obtained similar results to Zondek and Aschheim (1928)

Recently, it was reported (Hill and Parkes, 1931) that ovulation could be induced with ease in both normal and hypophysectomised (by decerebration) pregnant rabbits, and in view of the contradictory nature of other workers' results on the rat and mouse, it seemed that our acute experiments on pregnant rabbits should be followed by others designed to study the effect of induced ovulation on the fœtuses, especially in view of the reports that excessive luteinisation of the ovaries leads to disturbance of the parturition mechanism (Teel, 1926 , Evans and Simpson, 1929)

As the experiments described in the present paper were being completed, Wolfe (1931) published an account of somewhat similar work This author induced ovulation in 10 pregnant rabbits , 7 were killed within 30 hours of injection, and the remaining 3 at 10, 11 and 13 days after injection No disturbance of the fœtuses or placentæ was observed in any of the animals, Even more recently, Snyder and Wislocki (1931) have reported that ovulation can be induced, but that the fœtuses are often, though not necessarily, disturbed They have also (Wislocki and Snyder, 1931) obtained fertilisation of a second set of ova in the presence of existing blastocysts, a condition of affairs which they optimistically refer to as " experimental superfœtation "

The work described in the present paper was designed to study the effect of induced ovulation on the existing fœtuses and on parturition, and to determine the life history of the two sets of corpora lutea

II *Technique*

Extracts were prepared as described previously, injection being into an ear vein In many of the initial acute experiments, dated pregnancies were not available , in such cases a close approximation was obtained later by examination of the fœtuses

Detection of Ovulation —Detection of ovulation presented no difficulty till 10–12 days after injection Up to this time the ruptured follicles and young corpora lutea are quite distinct from the existing corpora lutea of pregnancy. Subsequently, however, the two sets tend to approximate, especially since the second set appears to undergo retrogression earlier than do corpora lutea of pregnancy In these conditions recognition may be difficult Vital staining of the existing corpora lutea with Chicago Blue was attempted, but this involved waiting 4–5 days for the dye to clear out of the serum before the new ovulation could be induced, and the technique considerably increased the risk of manipulative disturbance of the pregnancy Exploratory laparotomy or unilateral ovariectomy after injection was not resorted to, although this technique was successfully used by Wolfe From 10–12 days after injection onwards, histological examination of the ovaries was necessary Study of the cell fat changes, etc , was carried out as previously described (Deanesly, 1930)

III *Induction of Ovulation in the Pregnant Rabbit*

Thirteen rabbits from 7–29 days pregnant were injected with extracts of urine of pregnancy and killed 15–36 hours later Of these, seven ovulated from one or more follicles, the largest ovulation obtained being 12 follicles in a 29-day pregnant animal Within the range of pregnancies used, the ease with which ovulation could be induced showed no noticeable correlation with either the stage of the pregnancy or the number of existing corpora lutea In these initial experiments no attempt was made to study the effect on the fœtuses , any effect in the short time of observation would be limited to immediate abortion Subsequently, 26 pregnant rabbits were injected with untreated urine of pregnancy, with the special object of observing the effect on the fœtuses of ovulation and the presence of a second set of corpora lutea Of this batch, 21 ovulated The five negative animals all received only 2 c c or less of untreated urine Seven control non-pregnant animals receiving between 2-5 c c of urine all ovulated, 24 ruptured follicles being found in one receiving 3 c c On this evidence, it is not appreciably more difficult to induce ovulation in pregnant than in non-pregnant animals On the other hand, the experiments emphasise a fact to which we have previously called attention (Hill and Parkes, 1931), *i e* , the comparative inefficiency of our extracts as compared with untreated urine in causing ovulation in the rabbit, as contrasted with their much greater efficiency in causing the typical dual reaction in the immature mouse

IV *Effect on the Fœtuses of Ovulation and the Formation of a Second Set of Corpora Lutea*

The 21 pregnant rabbits ovulating after injection of untreated urine were killed from 3 days after injection up to what would have been full term or later The results are summarised in Table I

Of the 15 animals killed before full term, 4, killed at 3, 4, 7 and 9 days after injection, contained normal living fœtuses only, while 2, killed at 3 and 6 days after injection, contained living fœtuses in conjunction with reabsorptions.

Table I—Effect on the Fœtuses of Inducing Ovulation during Pregnancy

No of animal.	Days pregnant at injection.	Urine given (c.c.).	Autopsy (days after injection).	Corpora lutea					History of the fœtuses.
				Of pregnancy		Of new ovulation.			
				Age (days)	Diameter (mm)	Age (days)	No	Diameter (mm)	
APG 48	21	10	9	30	1 8 (1)	9	6+5	1 8 (2)	Reabsorbing at autopsy
APG 50	17	10	6	23	1 8 (1)	6	8+4	1 9 (1)	Reabsorbing at autopsy
APG 51A	19	5	4	23	—	4	6+6	—	Two normal living fœtuses at autopsy.
APG 55	27	2	3	30	—	3	Number	-	Fœtuses aborted 2 days after injection.
APG 60	17	3	6	23	—	6	4—7	-	One normal living fœtus, some pre-injection reabsorptions
APG 61	17	2	3	20	—	3	5+9	—	Four normal living fœtuses at autopsy
APG 63	16	1	9	24	2 5 (2)	9	2+1	1 8 (2)	None normal living fœtuses at autopsy.
APG 64	19	3	11	30	—	11	Large number	—	Advanced reabsorption at autopsy
APG 65	18	3	12	30	—	12	Number	—	Advanced reabsorption at autopsy
APG 67	21	2	7	28	2 4 (1)	7	2+3	2 0 (1)	Six normal living fœtuses at autopsy
APG 68	21	2	3	24	—	3	5+3	—	One living, one dead fœtus at autopsy
APG 69	24	2	4	28	—	4	2+1	-	Three aborted 4 days after injection. Three living, one dead at immediate autopsy
APG 72	15	1	11	26	—	11	2+3	-	Reabsorbing at autopsy
APG 78	14	1 5	3	17	—	3	Number	—	Aborted 3 days after injection.
APG 81	c 15	1	16	31	1 6 (1)	16	2+1	2 4 (1)	Two fœtuses still-born 16 days after injection, three more dead inside.
APG 82	c 10	1	23	c 33	—	23	Number	—	Post-term fœtuses with soft parts beginning to reabsorb found at autopsy
APG 83	22	1 5	2	24	—	2	4+5	—	Aborted 2 days after injection.
APG 85	16	1	15	31	1 6 (2)	15	Number	2 5 (2)	Gave birth to full-term dead fœtuses.
APG 86	16	1	15	31	1 7 (4)	15	Number	1 8 (3)	Gave birth to one full-term live fœtus and some dead
APG 89	15	1	12	27	—	12	Number	—	Reabsorbing at autopsy
APG 91	20	2	6	28	1 5 (2)	8	11+10	1 6 (1)	Partial abortion 2 days after injection, rest reabsorbing at autopsy

In 3, the pregnancy was terminated by abortion within 3 days of injection. In 4 others autopsy showed that the fœtuses were undergoing reabsorption, probably dating from the injection The effect produced shows little or no correlation with the dose given or the number of follicles ovulating One rabbit aborted one fœtus 2 days after injection and reabsorbed the rest The remaining animal aborted two fœtuses 2 days after injection, and, being killed immediately, was found to contain two normal living fœtuses and one dead

Six animals were killed at term or later Two of these injected at 18–19 days pregnant respectively, had almost completed the reabsorption of the fœtuses The remaining four carried the fœtuses to full term or later APG 86 gave birth at 31 days to one live fœtus and a number of dead ones, while the litter of APG 85, also born at 31 days, were all born dead but fully developed APG 81 and 82 were not accurately dated pregnancies The former aborted two full term fœtuses 16 days after injection, while two more dead fœtuses were found *in utero* APG 82 failed to show any signs of parturition 23 days after injection, and on autopsy was found to contain three large dead fœtuses, which judged by their size and the growth of hair, appeared to have lived beyond full term, but which were now showing signs of reabsorption of the soft parts

It may be concluded that the induction of ovulation is often compatible with continued development of the existing fœtuses, judged by our experiments, however, there may be some subsequent disturbance of the normal parturition mechanism, due possibly to the presence of a second set of corpora lutea

V *Histology of the Corpora Lutea following Ovulation Induced during Pregnancy.*

The corpora lutea from the ovaries of APG 48, 50, 63, 67, 81, 85, 86 and 91 were examined and measured in section after fixation in Flemming and Bouin In APG 50, 63 and 91 two corpora lutea, 6–9 days old, resulting from induced ovulation were of the type found in the first 10 days of pregnancy, though their cells and mean diameters were smaller, they contained a regular light granulation of osmicated fat The original corpora lutea of pregnancy in these animals contained diffuse osmicated fat in irregular granulation, typical of old corpora lutea Both from the appearance of the fat and from the size (diameters 1 5 mm, 1 8 mm, and 2 5 mm), these corpora lutea appeared more degenerate than those of corresponding normal pregnancies of a similar age Histological changes in the corpora lutea during the last half of pregnancy, consisting mainly in the gradual disappearance of osmicated fat from sections of Flemming fixed material, have been described in an earlier paper (Deanesly and Parkes, 1931).

The corpora lutea from the induced ovulation in APG 48 and 67 (9 and 7 days old respectively) differed from the corresponding ones described above, since in section they already showed signs of decay The outer lutein cells contained a regular granulation of osmicated fat, but none was present in the interior of the corpus luteum More osmicated fat was present in APG 67 where the induced corpora lutea were 2 days younger than in APG 48

The old corpora lutea of pregnancy in APG 67 showed blurred irregular fat beginning to disappear from the inner region. In APG 48 the corpora lutea of pregnancy contained only traces of osmicated fat at the edge

In APG 81, 85 and 86 killed 15 and 16 days after the injection of urine of pregnancy, none of the numerous corpora lutea in the ovaries appeared functional By examination and measurement of sections, however, two types of corpora lutea could be distinguished, the old corpora lutea of pregnancy being more shrunken and fibrous than those resulting from the induced ovulation In both types the osmicated fat had disappeared from most of the corpus luteum and tended to form a narrow peripheral rim Examination of the above material suggests that the corpora lutea developing after an induced ovulation pass through the normal histological changes more rapidly than corpora lutea of pregnancy and, though varying in size, they never reach the maximum diameter attained by the latter (2 9 mm) Their presence appears to accelerate the shrinkage and decay of the corpora lutea present at the time of injection, a finding difficult to correlate with the disturbance of parturition

VI *Summary*

(1) Ovulation can be induced with ease in the pregnant rabbit by the injection of urine of pregnancy

(2) A second ovulation is often compatible with the continued development of the existing foetuses, though some subsequent disturbance of the normal parturition mechanism may occur

(3) The corpora lutea formed after the induced ovulation have a shortened life history as compared with normal corpora lutea of pregnancy, and appear to exercise an adverse influence on the existing corpora lutea.

We are much indebted to Professor J. P. Hill, F R.S , for assistance with the examination of foetuses, and to Miss R Deanesly for the notes on the histology of the corpora lutea.

The expenses of the work were defrayed from grants to A S.P. from the Medical Research Council and the Foulerton Committee of the Royal Society.

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The Analysis and Synthesis of Some Diseases of the Mosaic Type. The Problem of Carriers and Auto-Infection in the Potato

By Redcliffe N Salaman, M D Camb, Director, Potato Virus Research Institute, Cambridge

(Communicated by H H Dale, Sec R.S —Received December 10, 1931.)

[Plates 6–9]

Recent work on the virus diseases of the potato has not only led to a reconsideration of the problems of the relation between virus infection and the clinical picture of disease, but has furnished us with evidence as to the existence of two distinct viruses and their co-existence in certain diseases whether patent or carried

The present work deals with still another, a third virus element, the analysis and the synthesis of certain virus diseases from these elements, and the relation subsisting between the viruses when present together within one and the same plant

The occurrence of the spontaneous splitting of a virus complex both in an actively diseased plant as well as in a carrier, is described, and evidence produced in support of the view that two or more virus elements within a potato plant may become linked together in such a manner that the resulting reaction they produce is peculiar to the specific type of complex created, and cannot be referred to that of a mere mixture of virus elements.

A short sketch of the steps by which this attitude towards virus infection of the potato has been reached will be of assistance to an understanding of the problems about to be discussed

The same Clinical Symptoms can be the expression of Distinct Diseases.

(*a*) The discovery of para-crinkle and its relation to Crinkle " A " (Salaman, 1930, *a*, and Salaman and Le Pelley, 1930)

(*b*) The observation that the same type of streak reaction, namely, the top-necrosis of Quanjer (1931) may be due to different virus complexes (Salaman, 1930, *b*)

Different Methods of Infection may give rise to different Clinical Reactions

(*a*) The observation that grafting and needle inoculation of Crinkle " A " produces entirely unlike results in healthy plants was first observed by the writer in 1928 (Salaman, 1930, *a*)

(*b*) The observation by Dr Kenneth Smith (1929, *a*) that needle inoculation and aphis infection from the same clinical disease may give rise to entirely different reactions on healthy plants

A Single Virus may be extracted by an Aphis from a Complex which the Needle conveys as a whole

This was demonstrated by Dr Kenneth Smith (1930) in regard to a mosaic disease in Arran Victory and later in respect to several other diseases of the mosaic group

Virus Diseases may be modified by passage through other Solanums

(*a*) The writer and Le Pelley (Salaman, 1930, *a*, and Salaman and Le Pelley, 1930) showed that passage of the viruses of Crinkle " A " or para-crinkle through an intermediate Datura scion on to a healthy potato plant gave two distinct classes of results according to whether the Datura was leaved or leafless

(*b*) Dr Kenneth Smith showed that one virus, namely, his X virus, increased in virulence by passage through tobacco and was attenuated by passage through Datura

Viruses may be separated by the Selective Action of certain of the Solanaceæ

Dr Kenneth Smith (1931, *b*) showed that his Y virus was held up on inoculation to Datura and his X virus on inoculation to Petunia, whilst the former was accepted by Petunia and the latter by Datura.

The Grouping of Virus Diseases and the Morbid Histology of the Reaction they induce

This was shown by Quanjer (1931) to hold good for certain classes of streak disease The writer (1930, *b*) however, found that one of the main groups allowed of considerable further division by the study of differential reactions in various hosts Further work by the writer and Mr F C Bawden (1932) on this subject is in course of publication

The Existence or otherwise of Intracellular or " X " Bodies in the Leaf Tissues of Affected Plants

Hurst (1931) has shown that in some of the mosaic infections, which have arisen in the writer's work, such can be found, in others not (see § 21)

Quanjer (1931) has pointed out similar facts in respect to some of the streak diseases

The Discovery and Isolation of Independent Viruses common to many Diseases of the Mosaic Group in the Potato

Two of these, namely, the X and Y viruses, were discovered by Dr Kenneth Smith , the third, the Z virus, by the writer, and it is the interaction of this latter with the other two which forms the subject of the greater part of this communication

It was the isolation by Dr Kenneth Smith (1931, *a*) of the two unit viruses which he has named X and Y, which put us in possession of a key to many of our problems The writer has great pleasure in acknowledging his debt to Dr Kenneth Smith, in that he has used his discovery to redirect the analysis of the different crinkles and streaks on which he has been engaged It was a coincidence that the study of the reactions of a certain crinkle in Epicure by the writer and his assistant, Mr F C Bawden, had led to the recognition of a virus with distinctive characters several months before we were aware of the existence of the Y virus as a separate entity It soon became obvious that we were dealing with a pure culture of the Y virus which Kenneth Smith was actively studying and which he had isolated by a beautifully adapted method of analysis With the new weapon—the X and Y viruses—in our hand, no time has been lost in reattacking the problems which were in hand, viz , the analysis and synthesis of virus diseases of the potato.

As it will be necessary in the course of this communication to use terms which may be unfamiliar to those unacquainted with virus diseases of the potato, a short description of the diseases as recognised clinically may be of value The virus infections of the potato may be grouped on a clinical basis as follows —

1 *Leaf Roll Group*—A disease characterised by the accumulation of starch in the leaves, which become thickened and longitudinally rolled, this is followed by stiffness and dwarfing of the stem and a great reduction of crop. The disease is reproduced in the plants raised from the tubers of an infected plant To this group belong also Marginal Leaf Roll, Rolled Edge, and Apical Leaf Roll The investigations here described do not deal with any of this group, nor do they offer any support to the view put forward by Whitehead (1931) that the crinkle in Field Marshal, which he considers is a product of the combined infection of mosaic and leaf roll, is of the same nature as the crinkle disease which will be discussed in these pages

2 *Mosaic Group*—This includes —

(*a*) *Mild Mosaic*—Plants which are more or less normal in stature, texture and yield, but which exhibit either permanently or for a time—generally the earlier part of their seasonal growth—a mottling of the leaf, which may be accompanied by a slight waving or ruffling of the leaf surface

The mottling may be —

(1) *Veinal mottle*, pale areas appear on a leaf vein, usually at a junction with a branch vein, and may spread thence to the areas immediately surrounding The mottle begins as a clearing of the veins

(2) *Interveinal mottle*, pale areas lie in between the major veins, such areas are variable in size

(3) *Mixed mottle*, pale areas on one and the same leaf may be either closely related to the veins, or situated on the areas between the veins

The distinction between these several types is no absolute one as was supposed by earlier writers, though one or other may, and often does, predominate in particular infections

A further distinction is made between mild and severe mosaics, this differentiation is based on the degree of the concomitant ruffling of the leaf surface and waving of the leaf edge rather than on the brilliance or otherwise of the mottle itself

(*b*) *Crinkle.*—Affected plants may be considerably dwarfed and are brittle in the stem, the leaves are mottled, often brightly, their surface thrown into irregular bulgings and depressions, the edges waved and often deformed and twisted. The crop is markedly reduced in quantity, and the tubers in size.

There are at least three types of crinkle (1) Crinkle "A," which can be reproduced by grafting on nearly every variety Disease symptoms on the test varieties President and Arran Victory are taken as being its classica expression (2) Para-crinkle which, though clinically akin to Crinkle "A," is a distinct disease latent in President and very pronounced in Arran Victory There are other clear-cut reactions which further differentiate these two crinkles, for which the reader is referred to the original papers (Salaman, 1930, *a*, and Salaman and Le Pelley, 1930) (3) A crinkle which produces a simple crinkle in Arran Victory but a severe mosaic or even crinkle on President, accompanied by a necrosis along the veins, ending in the death of the leaf this we now know to be induced by the Y virus when acting alone

3 *Streak Group* —In this group the disease may first show itself as a mosaic or a crinkle, but there soon intervene necrotic lesions which may kill the plant outright There are two main types of streak (A) in which minute necrotic lesions appear on the smaller unfolded leaves of the growing points and spread downwards This is Quanjer's (1931) acronecrotic group This class of streak has been divided by the writer and his assistant, Mr Bawden (1932), into at least three distinct groups according to their varietal clinical reactions. (B) The acropetal group of Quanjer, in which the necrotic symptoms appear first on the intermediate or lower parts of the plant as producing streaks along the veins of the leaves which, drying up, hang by their petioles, producing the peculiar picture known as Leaf Drop Streak This particular type is the characteristic reaction of the Y virus in certain varieties

In order to present the results of this investigation in as brief and clear a manner as possible, it is proposed to set forth the tale in its logical sequence rather than in the order in which the data were actually secured

§ 1 When Crinkle "A," as existing in President, Arran Comrade, Arran Victory, Irish Chieftain, Champion, and Arran Banner, is grafted to healthy plants of the same varieties, a well-defined clinical disease similar to that displayed by the infecting plant is engendered in the stock plant in each case Of such grafts 42 have been fully recorded, whilst many others have been made for stock purposes

§ 2 If Crinkle "A" be communicated to healthy plants of these same varieties by means of needle inoculations instead of by grafting, a mosaic mottle, interveinal in character develops, and neither in the season of infection nor in subsequent seasons does the crinkle reappear, figs 1 and 2, Plate 6. These observations have been made on 46 different plants The mosaic-affected plants thus derived are, for convenience, here spoken of as examples of *Reduced*

Crinkle " A " One single exception has been observed to occur in the variety Arran Banner, which is now being studied

The same result follows if, instead of inoculation, a cutting of a healthy potato plant be immersed in the juice of an infected plant and allowed to suck up as much juice as it can in 3 to 4 days and then is grafted to a fresh healthy stock , there was no sign of crinkle, only a mild mosaic in 1929 , in both 1930 and 1931 the symptoms were those of a well-developed blotchy interveinal mosaic. The crinkle symptoms never reappeared (*cf* § 10)

§ 3. It is evident that the results of inoculation and of grafting the same virus disease, namely, Crinkle " A," produce constant but dissimilar results This distinction was first observed in 1928 , three possible explanations were then considered .—

(A) The distinction noted might be due to the extreme differences in the quantity of the initial dose Against this view is the lack of any parallel phenomena in either animal or plant virus infection, but still more adverse is the fact that if such mosaics derived by inoculation of Crinkle " A " be carried on to healthy plants by means of grafting, the result is the same whether the further transfer is made by graft or by inoculation, viz , a mosaic similar in character and reaction to that originally obtained Such transfers have been made by graft 18 times and by inoculation 12 times

(B) The virus might be reduced in virulence when inoculated by needle by reason of the oxidation of the infected juice, as Johnson and Ogden (1929) report to be the case with tobacco mosaic This latter might very readily take place when the juice is extracted by pounding up the plant tissues in an open mortar To check this, inoculations were made subcutaneously with a juice extracted anaerobically from the plant tissues by means of a high pressure syringe The results were the same—a mild mosaic of a mixed or of an interveinal type

(C) The virus present in Crinkle " A " is a mixture of several viruses of which one or more constituent is non-inoculable

It is this view which the writer believes to be the correct one. This so-called non-inoculability might be in reality due to the destruction of the virus in question through some procedure in the manipulation, but the experiment referred to in (B) would appear to exclude the most probable of such factors as might come into play.

§ 4 Employing the methods of recognising the viruses X and Y as described by my colleague Kenneth Smith, it was readily proved that the mosaic infection, observed in healthy plants after infection either by grafting or inoculation with Crinkle "A," invariably contained the virus X which produced its characteristic rings and mottle on tobacco and its peculiar mottle with green veinbanding on Datura It became apparent, however, that although all our Crinkle "A" plants were clinically very much alike, they were not all suffering from an infection of the same viruses Thus a crinkle in Myatt's Ashleaf, which was used for a time in 1928–29, has been conclusively proved by Dr Kenneth Smith (1930, 1931) to contain both X and Y, whilst in a crinkle in President, which forms the basic material of much of the work here described, as well as in a similar crinkle of Arran Comrade, no Y virus has been demonstrated by the writer either by repeated needle inoculation or aphis infection on tobacco On the other hand, a crinkle on Irish Chieftain which has been used to a considerable extent, and which can be transferred readily to President, behaves on inoculation in a precisely similar way to President-crinkle, yet it contains, like the Myatt's Ashleaf, both X and Y It is interesting to note that the crinkles in both Irish Chieftain and Myatt's Ashleaf, though similar in type to that of President, are more severe in their leaf deformity and brighter in their mottling than the latter, figs 3 and 4, Plate 6

§ 5 The X virus has been demonstrated in both the reduced and unreduced Crinkle "A" in many scores of tobaccos and Daturas, what, then, is the difference between reduced and unreduced Crinkle "A"? It is not merely a reduction of virulence in the X virus, because this reduced crinkle has been passed through two generations of tobacco and returned to potato and passed on to other potatoes, and in no case has the true clinical Crinkle "A" been regained. It is true, as Kenneth Smith has demonstrated, that X can vary considerably in virulence We shall refer to this later in § 10, but clearly the difference between reduced and unreduced Crinkle "A" is not due to this cause It may be permitted to anticipate the evidence to be adduced later (§§ 14–16) and answer this question by stating that there is another virus element, an uninoculable one, at work, which the writer has called Z which, in conjunction with X, is responsible for the Crinkle "A" as present in President We may therefore regard this type of Crinkle "A" as having a virus content which may be described by the formula ZX

§ 6 In the writer's previous paper (1930, *a*) a type of experiment known as double-grafts was described and results from its employment in two different virus diseases, Crinkle "A" and para-crinkle, recorded. In these experiments the infecting scion was grafted on to Datura and the Datura in its turn grafted to a healthy potato There were two types of the experiment one in which the Datura was furnished with leaves, and one in which the Datura had a bare stem The results in both cases with either virus group were similar, *i e*, Crinkle "A" and para-crinkle double-grafts with an intermediate leafless Datura scion reproduced the full clinical disease on the underlying potato stock, whilst, when leaves were present on the Datura, neither source of infection reproduced a crinkle, but either a mosaic or nothing These experiments were done in 1929, others have since been performed of the same kind but, what is more important, the underlying stocks have been grown on for a further 2 years, yielding results of much interest

§ 7 Before considering the facts referred to above, a further and new experiment should be here considered Kenneth Smith (1931, *b*) has shown, and the writer has confirmed the observation, that if some plants containing the Y virus be inoculated to Datura, the Y fails to make an entry Instead of inoculating the Y virus, the writer has made double-grafts with the Y virus as the exciting infection, and President as the basal stock The result is most striking, figs 5 and 6, Plate 7 If leaves are present on the Datura the Y fails to pass through the Datura and the basal plant is as healthy as if nothing had been done to it, if leaves are absent, the Y virus descends on to the President stock and kills it

§ 8. Now let us turn back to the behaviour of Crinkle "A" and para-crinkle We may for simplicity group the relevant facts as follows —

(*a*) Crinkle "A" double-grafts with leaves In the current season the basal stock, whether President or Arran Victory, develops a mosaic which is generally faint and more or less veinal.

(*b*) Seven such plants have been grown on and in all the symptoms have increased in intensity, five have definitely returned to a full Crinkle "A," and in the remaining two the mosaic is a severe interveinal one. Such plants all contain X

(*c*) Para-crinkle double-grafts with leaves. In the current season the symptoms on an underlying Arran Victory stock are those of a mild mosaic; on a President they are non-existent

(*d*) Eleven such plants have been grown on for a second or third season, and in no case have the symptoms become notably increased and no suggestion of a return to the original para-crinkle has been observed. Such plants have given no reaction indicating the presence of either X or Y.

§ 9 It would seem evident that the leaved Datura intermediate scion plays two entirely different rôles in the two cases In Crinkle "A" the Datura has depressed the virulence of the X to a variable but always considerable degree, from which in the subsequent tuber generations it recovers In para-crinkle a drastic and definite change has taken place from which recovery is impossible.

In view of the result of double-grafts (§ 7) on the Y virus, it would seem very probable that the Datura has removed a Y element from the para-crinkle virus complex completely

It might be objected that the virus Z is the one that has been depressed, and not the X It will be shown in § 17 that Z, when derived from a source altogether free from any influence of Datura, is not to be distinguished from one that has passed through a Datura-leaved double-graft (§ 15)

Moreover, when Z itself is passed through a similar Datura double-graft, it emerges on the basal Arran Victory very much the same as it entered

§ 10 The influence of Datura on the X virus can be gauged by comparing the two sections of Table I, where X was introduced into the healthy varieties in Group A by a previous passage through Datura on to Arran Victory and thence to the varieties stated , and in Group B by direct inoculation

It will be seen that in Group A the symptoms are slight in the first and more severe in the following season, whilst in Group B there is greater uniformity as between first and subsequent seasonal symptoms It should be remembered that in Group A the virus has had one passage through a potato after leaving the Datura except in the last example cited

If a scion containing the virus X alone be used as an infector by double-graft through a Datura with leaves, the healthy stock plant shows no sign at all of disease, so strong is the effect of the Datura juice in depressing the action of the X virus In the next season, however, the stock develops a definite interveinal mosaic This result is strictly comparable to the first seasonal effects of double-grafting with Crinkle "A" mentioned in § 8

§ 11 The para-crinkle complex is a peculiar one . it has been inoculated into a hundred or more Datura and tobacco plants without producing any reaction * Whilst it can be communicated by grafting into several varieties producing a more or less serious crinkle, it cannot be inoculated into any Dr Kenneth Smith has shown by means of aphis inoculation that para-crinkle contains Y, which finding receives further support from the evidence of the Datura double-grafts (§ 8), but there is no evidence of any X being present

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* On Datura certain exceptional and very mild symptoms were noted in a few cases in a previous communication (Salaman and Le Pelley, 1930).

Table I

Variety	1929		1930.		1931	
	Pot No	—	Pot No	—	Pot No	—
			Group A.			
Arran Victory	399 A1	Definite mosaic	—	Well developed interveinal mosaic		
,,	399 A4	Definite mosaic	—	Very severe inter veinal mosaic		
President	399 P2	Very mild mosaic	517	Severe inter veinal mosaic	213 214	Severe inter-veinal mosaic Almost a crinkle
,,	399 P4	Mild mosaic	518	Severe inter veinal mosaic	215	Severe inter-veinal mosaic
Abundance	399 Ab	Very mild mosaic	—	Mild mosaic		
Arran Comrade	399 ACde	Definite mosaic	-	Severe inter-veinal mosaic		
Sharpe's Express	399 ShE	Very mild mottle	—	Mild interveinal mosaic		
,,	151 ShE	Mild mosaic	—	Mild interveinal mosaic		
,,	401	Mild mosaic	585	Severe inter-veinal mosaic		
President	850 4P	Very mild mosaic	811	Mild interveinal mosaic	226	Mild interveinal mosaic
,,	250 Dat P3	Mild mosaic	560	Severe inter veinal mosaic	707	Interveina mosaic
			Group B			
Arran Victory	248 A2	Definite mosaic	514	Definite inter veinal mosaic	167	Interveinal mosaic
President	248 P1	Definite mosaic	517	Definite inter veinal mosaic	169	Mild interveinal mosaic
,,	248 P_1A_1	Definite mosaic	519	Mild interveinal mosaic	170	Mild interveinal mosaic
,,	248 P_1P_1	Mild mosaic	520	Severe inter veinal mosaic	171	Mild interveinal mosaic
,,	248 P_1P_4	Definite mosaic	521	Severe inter veinal mosaic	174	Very mild mosaic
Arran Comrade	248 A Cde	Apparently healthy	529	Very severe interveinal mosaic	183	Severe inter-veinal mosaic
Abundance	248 Ab	Definite mottle	530	Very mild inter veinal mosaic	530	Very mild inter-veinal mosaic

It is clear that the disease para-crinkle is not merely due to Y, for that is most readily inoculable and reacts most violently in President and with less severity on Arran Victory. Para-crinkle, on the other hand, is carried harmlessly by President* but produces a most severe crinkle on Arran Victory.

* Occasionally a veinal clearing or veinal mottle develops and then dies away

It has been mentioned (§ 6, c) that in those Datura double-grafts with leaves, when Arran Victory is the stock, a mosaic ensues which is evidence that something else besides Y is present It will be shown that this mosaic is produced by a separate virus, which we have good reason to believe is the same as that hypothecated as Z in § 5

It should be added that the deduction that para-crinkle is induced by a complex of Z and Y is in general harmony with all the experimental data except in one case When a healthy Arran Victory shoot was allowed to suck up para-crinkle juice in a manner similar to that described in § 2 for Crinkle " A," the result was a mottling which in the two subsequent seasons' growth from tuber settled down to a definite interveinal mosaic Till further work on these plants has been done it is difficult to harmonise this result with its fellow from Crinkle " A " and the formula ZY It might be that we are dealing here with a specialised type of the Y virus and this might explain the failure experienced in the attempts to synthesise para-crinkle by merely bringing Z and Y together (§ 30) To indicate this the Y of para-crinkle will be written Y'.

The behaviour of the Y virus from certain King Edward plants both in the glasshouse and the field lends further support to this view

§ 12 Although it is true that para-crinkle is a relatively stable complex, yet close observation of a large number of affected plants of the variety Arran Victory over several years has led to certain interesting deductions If a number of tubers from one and the same para-crinkle infected plant grown in the glasshouse be put out in the field plots, it has been frequently observed that not all the tubers give rise to para-crinkle plants (In the field a para-crinkle infection is in general so severe that the plant assumes the peculiar form known as Curly Dwarf) Often a normal or a mildly mottled plant occurs amongst others more severely affected No particular importance at first was attached to this variation of symptoms, for the appearance of which so many environmental factors might be held responsible It was not until three cases of a similar but far more decisive character were observed in the glasshouse that its importance was recognised.

§ 13 In 1930 several para-crinkle infected Arran Victory plants were raised from tubers descended from a single Arran Victory on which the carrier King Edward had been grafted in 1929 , these plants displayed the normal disease characters of para-crinkle In 1931 three more plants were raised, two from one and one from a second of the 1930 stock plants In each of the three 1931 plants one shoot was observed which was free of all crinkle and was either healthy to all appearances or at most exhibited a mild mosaic. As it was in one only of these plants that the appearance of the abnormal shoot was observed and studied from its first emergence, the consideration of the other two will be deferred to another occasion

§ 14 The seed tuber of the 1931 plant to be described originated from pot 606 in 1930 and was numbered 250 in 1931 It was planted in the pot 250 on March 16 and the peculiar shoot was observed on April 21 and photographed, fig 7, Plate 7 On that date there was a fully crinkled shoot 10 inches high and an apparently healthy one 4 inches high on which, however, the veins were slightly cleared In order to make sure that there was no accidentally introduced second seed tuber, the mother tuber was taken out, washed and photographed, fig 8, Plate 7 It will be seen that the "healthy" shoot is an integral part of the whole For the sake of convenience this shoot will be referred to as the N shoot, and the crinkled shoot as the D shoot On May 5 the N shoot exhibited a mild veinal mottle, this, however, soon disappeared and from then onwards the N shoot developed as an apparently healthy Arran Victory plant The crinkled D shoot matured considerably earlier than the N one, but both were harvested at the same time under water The relations of both stems to the remains of the mother tuber were still visible and their crops, apparently normal Arran Victory tubers, were harvested separately Grafts from the N shoot were made to Arran Victory and other varieties, and a rapid study was made of some of the reactions of the virus which it contained, these are detailed in § 15

§ 15 Reactions of the virus present in the N shoot of plant 250/31 under glasshouse conditions

Table II

Varieties infected	No of plants infected		
	By graft	By inoculation	
Arran Victory	5	—	In about 14 days the veins begin to clear and this develops into a definite mottle which at first is confined to the veins and particularly to those points where they divide The veinal mottle is sometimes accompanied by a slight ruffling of the leaf surface, but there is neither waving, deformity, nor necroses. About 6 weeks after grafting the symptoms tend to disappear, the mottling becomes mixed or even definitely interveinal, and the plant as a whole healthy. There is neither dwarfing nor stiffening of the stem or petioles.

Table II—(continued)

Varieties infected	No of plants infected		
	By graft	By inoculation	
Arran Victory	—	2	No reaction followed inoculation.
President	3	-	No reaction The scion grew vigorously and although the stock at first might look a little "suspicious," it soon regained its full colour and remained perfectly healthy
"	—	2	No reaction followed inoculation
Di Vernon	1	—	No reaction, scion good
Epicure	1	—	No reaction, scion good
King Edward	1	-	No reaction, scion good
Uptodate	1	—	No reaction, scion rather poor
Tobacco	-	5	No reaction
Datura		5	No reaction
Double graft through Datura with leaves on Arran Victory	1	—	A transient but definite clearing of veins in the Arran Victory stock, otherwise no reaction

§ 16 In the virus present in the N branch we are dealing with a constituent of the para-crinkle complex which is clearly neither Y nor X, that it is a companion virus to Y in para-crinkle is obvious and that it might be so to X in Crinkle "A" was a legitimate inference The writer has called it the Z virus.

The proof of this proposition has been attained by analytic methods in the case of para-crinkle and by synthetic ones in the case of Crinkle "A" To deal with para-crinkle first

§ 17 It has already been mentioned, § 9, that para-crinkle when passed through a leaved Datura graft loses what appears to be its Y′ and the resultant mosaic is permanent, fig 9, Plate 8 This residual mosaic, veinal in the earlier part of the season, interveinal later as is that in the N branch of plant 250, reacts, so far as it has been tested, in an exactly similar manner to the virus contained by the latter Theoretically it should be identical, for if we are right in assuming that para-crinkle is a mixture of Z and Y′ (see § 11), and we remove the Y′, we are obviously left with the Z Moreover, the virus existing in the N branch demonstrably contains neither X nor Y′, it should therefore correspond to what remains in the para-crinkle complex after Y′ has been removed

§ 18 Before proceeding to the consideration of synthetic experiments with the virus elements Z, X and Y, it may be well to supplement the evidence already given by Kenneth Smith (1931, *a*) as to the reactions of the two viruses X and Y The writer has had occasion to study both on a large number of the domestic potato varieties, and the reactions can be grouped into rather well defined classes They are detailed below for the convenience of other workers

It should be said that three sources of X virus have been employed two where the X virus has been the virus present after inoculating Crinkle "A" in President to Datura and grafting the Datura to healthy Arran Victory and President stocks respectively The third consisted of a strain of reduced Crinkle "A," *i e*, the resultant virus in Arran Victory or President obtained after inoculating Crinkle "A" from President into either of these varieties. The results in either case are identical, *cf* § 10, Table I

The Y virus as regards two sources has been obtained from Dr Kenneth Smith, whilst a third was the virus present in the Epicure crinkle which was mentioned on p 188 All three sources have reacted in an identical manner.

§ 19 The varietal reactions in the potato under glasshouse conditions which follow infection with the X virus may be grouped as follows —

No 1 No reaction or an almost imperceptible one—

Uptodate, Great Scot

No 2 Interveinal mosaic of varying degree—

Abundance, Arran Chief, Arran Comrade, Arran Victory, British Queen, Champion, Eclipse, Edzell Blue, Irish Chieftain, Kerr's Pink, Majestic, May Queen, President, Sharpe's Express

No 3 Acute top necrosis streak—

Arran Crest, Epicure, King Edward

The stock of Uptodate which was tested has been used several times and found to be free from any ascertainable virus, healthy looking Uptodate stocks are commonly infected with a virus complex which contains both the X and Y virus The lack of reactions to a pure infection of X, whilst not surprising, must not, however, be accepted as evidence for believing that a variety, which may happily carry a complex containing a specific virus, cannot respond to an infection with a specific virus contained in that complex

§ 20 The varietal reactions in the potato under glasshouse conditions which follow infections with the Y virus may be grouped as below :—

(1) No reaction—

Dr Vernon, Kerr's Pink

(2) Necrotic lesions only—

Veinal necroses only—

International Kidney.

Sharpe's Express

Veinal necroses and leaf drop streak—

Arran Consul

Arran Crest

(3) Mosaic lesions only—

Mosaic or crinkle with or without minute and inconsiderable necrotic spots—

Arran Victory
Duke of York
Epicure
King Edward

(4) Mosaic and necrotic lesions—

Mosaic, and veinal necroses—

Abundance
Arran Chief
Great Scot

Mosaic, veinal necroses and leaf drop streak—

Arran Banner
British Queen
Uptodate

Crinkle, veinal necroses and leaf drop streak—

King George
Majestic
President

In Group (1) occurs Dr Vernon, the stock of which has been proved to be carrying Y and very probably Z Here we have an absence of reaction reminiscent of a similar negative result when X is introduced to Uptodate (§ 4) On the other hand, our stock of King Edward is carrying para-crinkle which contains Y′, yet a further dose of Y brings about a sharp reaction The same is probably true for most stocks of Uptodate *

§ 21 The leaves of plants infected by the virus entities X, Y and Z, and many of their combinations, have been examined cytologically by Dr and Mrs C. C

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* Plants of a stock of the variety Uptodate in which no evidence of any contained virus has been found after repeated test over 3 years, as well as plants of a stock of the same variety in which a top necrosis complex proved to contain both the X and the Y virus is carried without symptoms, have both been inoculated with pure Y virus. In each case the plant went down with a lethal leaf drop streak. These facts are taken as additional evidence that a complex is a substantive group with properties peculiar to itself, and not a mere mixture On the latter hypothesis it would be difficult to understand how a plant already carrying without reaction the Y virus, should be stricken down by a further infection of the same virus.

Hurst and the writer (1931). Confining ourselves only to the presence or absence of the cell inclusions or " X bodies " we have found that —

In 20 plants containing the X virus only, X bodies were found in 16
In 13 plants suffering from Crinkle " A," X bodies were found in all
In 7 plants containing the Y virus only, no X bodies were found
In 2 cases where X and Y were present, X bodies were found in both
In 5 plants containing the Z virus only, in 4 no X bodies were found, and in 1 very rare and somewhat doubtful X bodies were found This result occurred in the plant 252, the residue of a double-grafted para-crinkle

The cytological findings thus provide further differential criteria which support the view that we are dealing with three distinct virus elements

§ 22 The characters of the three viruses having been established, it follows that—

The Z virus may be distinguished from either the X or Y by the following characters —

The Z virus is non-inoculable, whilst X and Y are both freely inoculable to the potato
The Z virus causes no symptoms in President, whilst both X and Y, and particularly the latter, produce most striking lesions in this variety
The Z virus produces no reaction by inoculation in either tobacco or Datura, whilst X causes symptoms in both, and Y a characteristic lesion in tobacco
The X virus kills Epicure, King Edward, and spares Uptodate; the Y virus reacts on the same three varieties with a crinkle or streak, whilst the Z virus spares all three
The Z virus by itself has but a very small range of activity, but in company with X or Y its reactions are manifold It might be described as a complementary virus

The writer is aware that objection can be raised to the claim that the Z virus is strictly a unit virus, but its claim to be so regarded does not fall behind that of either X or Y; further work may substantiate or cancel the rights of all of them. To-day we may say of all three that they are stable and react as single units That we have in Z a virus which is neither X nor Y is certain. What its and their exact status are must rest awhile in doubt

Whilst it is the opinion of the writer that the three viruses X, Y and Z acting singly or together are sufficient to explain the majority of the virus problems so far encountered in the potato, he would most expressly guard against the assumption that there may not be still others awaiting identification

§ 23 The existence of three separate viruses, X, Y and Z, having been established, we may now pass on to the consideration of the possibility of building up from them complexes comparable to or identical with those, such as interveinal mosaic, Crinkle "A," or para-crinkle, which have been the subject of considerable study in the past Such a synthesis as is here indicated has been attempted by bringing the elements together by graft or inoculation or both This method has many objections It enjoins uncontrolled dosage of any one of the entrant viruses, permits of but a minimal control of their respective virulence, and takes but little regard of the route by which the viruses attain their measure of dispersion throughout the plant tissues That so considerable a degree of success has been attained by such crude methods is in itself instructive as to the nature of the virus complexes as we know them both in the field and the glasshouse

The attempts at synthesis will be described in terms of the virus elements engaged All cases where grafts were not satisfactorily established have been omitted from this account

§ 24 *The Synthesis of X and Y* —In nature we have yet to be quite sure that plants occur with this virus content Certain interveinal mosaics from the field may be examples of such, but, as the Z virus was not recognised till 1931, those cases of interveinal mosaic in potatoes analysed by the writer and independently by Dr Kenneth Smith and found to contain X and Y cannot be fully accepted till the presence or absence of Z is demonstrated , inasmuch as one of these interveinal mosaics is inoculable as such and does contain both X and Y, we shall be probably quite safe in regarding it as free of Z and as an example of XY infection

Infections which must be regarded as XY in composition have been made artificially The writer in 1929 inoculated Irish Chieftain Crinkle "A" which contains both X and Y, as shown by tobacco inoculation, to healthy potato plants, and produced a mild mixed mosaic which theoretically should be consequent on X and Y in the first season, and a mild interveinal one in subsequent ones The appearance of these plants in no way differed from the reduced Crinkle "A" of President which contains X only. Dr Kenneth Smith (1929) inoculated healthy potato plants with a virus complex, derived from a mosaic infection of Arran Victory, which he subsequently identified as consisting of the X and Y viruses. These, after passage through tobacco, he returned to potato plants and induced on the younger leaves a severe mosaic of a bright and rather massive interveinal type with a variable amount of waving and rugosity On the older leaves necrotic spots were not uncommon. He observed the occasional retrogression, apart from temperature effects, of these severer symptoms to those of the original mild potato mosaic from which the experiments in the tobaccos originated , he further found that this mosaic could be readily transmitted to other potatoes by needle, but failed to do the same with the aphis *Myzus persicæ* In the following season Dr. Kenneth Smith (1929) found the progeny of these infected potatoes reproduced, without variation, the symptoms of the preceding year Potato plants artificially infected from tobaccos in which the virus element, probably the X virus only, has increased in virulence, do not in reality correspond closely to the diseased potatoes of the field On the other hand, the experience of Dr Kenneth Smith with interveinal mosaic in President corresponds with that of the writer It contains both X and Y and is transmissible by needle to other potatoes, but whether it contains the further virus element Z, though very unlikely, remains to be proved This particular source of interveinal mosaic occurred in the field and was given to the writer by Professor Paul Murphy

The evidence to hand suggests that the complex XY as it occurs in the field is identical with the reduced Crinkle " A " of Irish Chieftain as exhibited in Arran Victory or President as a result of artificial inoculation, and that the symptoms are those of a rather mild interveinal mosaic indistinguishable from those consequent on a simple X infection

§ 25 *Artificial Union of X and Y* —This was attempted 22 times, of which 14 only have been allowed to rank, as the Y virus in the source employed in the remainder had probably not become systemic at the time it was employed In some cases the X and Y either singly or together were brought in to the healthy plant by inoculation , in others they were both introduced by grafting, or one was grafted, the other inoculated In 9 out of the 14 host plants the Y virus " took charge," *i.e* , it produced its typical necrotic reaction in President or a crinkle in Arran Victory before any evidence of combination was visible Of the remaining 5, all developed a well-marked interveinal mosaic with occasional necrotic spots on the leaves It should be noted that these 5 possibly successful syntheses are indistinguishable from plants resulting from a pure X infection This in itself is no evidence against their being of the virus composition XY, but rather the contrary , it does, however, encourage a measure of caution till more positive evidence of their constitution is to hand.

§ 26 *The Synthesis of X and Z* —Plants affected with this complex occur plentifully in the field, several have been already described by the writer as examples of Crinkle "A," notably those in President, Arran Victory, and Arran Comrade The appearance and reactions of crinkle in these varieties have been described in detail in a previous communication (Salaman, 1930, *a*) Certain features may be recalled In both varieties the disease begins in the upper portion of the plant by a clearing of the veins which develops into a blotchy veinal mottle, the surface of the leaves becomes coarsely rugose and, in President, the outline of the leaf distinctly indented and waved, figs 1 and 3 Plate 6 In Arran Victory the waving and rugosity are less pronounced fig 10, Plate 8 As the season progresses the leaf symptoms spread to all parts of the plant and in President the deformity may be considerable, still later both the rugosity and mottling become less pronounced In Arran Victory a characteristic glazing and large waved ruffling of the older leaves sets in and to a considerable extent hides the previous crinkle symptoms, fig 11, Plate 8

§ 27 *Artificial Synthesis*—This has been effected in two ways -

(A) By the addition of Z to X

Pot No 252/171/P/31—The Z used is the residue from a Datura with leaves double-graft para-crinkle In this case both sources Z and X were introduced into a healthy President plant by grafts The result was a typical crinkle which developed in exactly the same manner as does a normal crinkle in a healthy plant after a straight graft with Crinkle "A," the only difference being that in this case it took some 3 weeks longer for the full symptoms to appear

Pot No 250/171/A/31—The Z used was derived from the N branch of the para-crinkle plant 250 Both Z and X were introduced by graft. In this case the Crinkle "A" symptoms in the Arran Victory stock plant were definite 6 weeks after grafting and developed quite normally

Pot No 250 A A /215/A/31—The Z virus was again derived from the N branch, but in this case had been passed twice by graft through Arran Victory Both scions were grafted to a healthy Arran Victory In this case a true crinkle was never fully developed, but the leaves developed a very severe mosaic when the plants matured

(B) The second method of building up Crinkle "A" was somewhat complicated It was used on two occasions, the only difference between them being that the healthy recipient of the viruses was an Arran Victory plant in one case and a President in the other.

Pot No. 559 K. Ed. Dat. A1/30.—A symptomless King Edward carrying para-crinkle was grafted with a reduced President Crinkle " A " which, as is usual in King Edward, produced a top necrosis streak. A shoot of this infected Edward was then used as the top scion on a double Datura-with-leaves-graft on an Arran Victory base. In the first season a very severe interveinal mosaic developed which passed into a mild crinkle. In the second season the progeny of this plant was a perfectly typical Crinkle " A " in Arran Victory, which towards the end of the season passed through the typical stages ending in the characteristic ruffling and glazing of the older leaves, fig 12, Plate 9

Pot No. 559 K Ed Dat P1/30 —There was no change of procedure except that the basal host plant was a healthy President The seasonal symptoms were those of Crinkle " A " of a rather mild type, in the following season, however, the progeny presented a characteristic picture of Crinkle " A " in President, fig 13, Plate 9

Grafts from the Arran Victory host plants were made to further healthy Arran Victory and President plants and in both Crinkle " A " symptoms developed normally

The two successful compositions here described may be interpreted thus. King Edward has a virus content due to its latent para-crinkle of ZY′, to it was added an X The mixture ZXY′ was then passed through Datura with leaves which extracted the Y′, and the remaining virus elements brought intimately together passed on to the healthy hosts to give rise to the train of symptoms which we have learned to associate in Crinkle " A " with the virus complex ZX.

Omitting other experiments where the grafts were poor or actually failed, it is permissible to say that five attempts to reconstruct Crinkle " A " have been made, and all have been successful

§ 28. *The Synthesis ZX and X* —No diseased condition of the potato has as yet been identified to which this composition might be attributed. Inasmuch as ZX is Crinkle " A," the addition of an X to an already crinkled plant would possibly not give any appreciable sign of its presence, though this would not rule out such a possibility if an actively crinkled scion were introduced simultaneously with an X in a neutral plant (see § 41). In the attempts here recorded to combine these viruses the ZX was the virus complex in a President plant resulting from a Datura-double-graft-with-leaves of Crinkle " A " in which the X had been attenuated by its passage through the Datura and not having regained its full expression the plant exhibited not a crinkle but an interveinal mosaic with a few necrotic spots on its older leaves

Three attempts were made --

Pot No 200/215/P/31 --Both sources were grafted on to a healthy President and both scions grew vigorously, the only real reaction on the host was a faint interveinal mosaic

Pot No 200/171/P/31 —Both scions were grafted to a healthy President and both grew well The host scarcely reacted at all except for a fleeting veinal mottle

Pot No 200/171/A/31 —The procedure was as above, but the host plant a healthy Arran Victory, the scions prospered, but the plant failed to react

These three attempts to combine ZX and X have thus ended in a species of stalemate So far from the virulent Crinkle " A," ZX, being intensified by an additional X, its action has been practically suppressed, whilst X, which invariably reacts on a healthy plant with a quite definite and often severe mosaic, has also been practically put out of action This phenomenon will be referred to again in § 39

§ 29 *Synthesis ZX and Z*—Whether such a virus grouping occurs in the field is not known, but inasmuch as the result of artificial synthesis is merely an intensification of the crinkle common to ZX in one case and is but a mild interveinal mosaic in the other, it would not be easy to recognise its existence by sight

Pot No 492/251/P —A scion from a fully-developed Crinkle " A " on President and one from the double-Datura-leaved graft residue of a para-crinkle, were grafted to a healthy President The result was an acutely crinkled plant which developed its symptoms considerably earlier and more rapidly than an ordinary Crinkle " A " grafted plant would do

Pot No 250A/195/A —In this case the Z was derived from the N branch of the para-crinkle 250 and the ZX was an active crinkle in President The scions matured early, especially the Z one, and the stock plant never developed more than a mild interveinal mosaic

Pot No 200/252/P —In this case the ZX in the attenuated form was derived from a Datura-double-graft with leaves of Crinkle " A " in President, whilst the Z was derived from the residue of a Datura-double-graft with leaves of para-crinkle Neither scions grew vigorously, and the result was, as in the case above, an interveinal mosaic

As in the two latter experiments the scions were not entirely satisfactory, no final conclusions can be drawn as to the effect of the juxtaposition of the viruses ZX and Z in the potato The highly intensified crinkle of the first experiment, however, is not unexpected when one remembers how Z adds a crinkle character to a mosaic producing virus such as X

§ 30 *The Synthesis of Z and Y* —In the field the disease produced by a complex which the writer believes to be ZY′ is the para-crinkle disease latent in every stock of King Edward in the kingdom,* and active in occasional plants of Arran Victory and Arran Chief. Its synthesis, if attempted by the method of simple juxtaposition of the virus elements, meets with a special difficulty at the outset Para-crinkle is more or less completely latent in President, a plant which is intensely susceptible to the Y virus, hence if we succeed in building up the complex in President and leave any Y over unattached it proceeds at once to kill off the host with streak If we use Arran Victory as the host plant we have a different difficulty The Y virus produces in Arran Victory a crinkle not so severe, but of the same type with the same preponderance of veinal mottling which is characteristic of para-crinkle The difference between the two is essentially one of degree helped out by the occasional presence of superficial necroses in stem and petiole in the case of para-crinkle If we succeed in building up para-crinkle in Arran Victory and leave any Y free, then we only expect an exacerbation of crinkle symptoms—a very intense para-crinkle and that, indeed, has been found to supervene in some cases Further, in order to establish a proof of successful para-crinkle synthesis, it is necessary that the virus complex produced shall arouse no symptoms on healthy President but excite a severe crinkle on Arran Victory

§ 31 *The Artificial Synthesis of Z and Y* —The Z was introduced by graft, the Y by inoculation in some cases and by graft in others In two cases where Arran Victory was the host the scion failed In one where President was the host both scions were poor, but as Y symptoms intervened after a quiescent interval of 6 weeks it is not impossible that next year we shall find some evidence of a latent para-crinkle

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* Over 150 different stocks of King Edward from different parts of England, Ireland and Scotland have been tested, and all except some half-dozen have given a positive para-crinkle reaction on Arran Victory Those which have so far failed are being tested again, it is not impossible, see §§ 14 and 43, that they are really free of the complex. I am indebted to Mr Millar, of the Department of Agriculture for Scotland, for testing a large number of these stocks.

These attempts may be dismissed as failures Greater success rewarded an effort which was prompted rather by intuition than logic In partnership with Mr F C Bawden, the writer has been investigating a number of streak-carrying plants One of these occurred in a unit of Di Vernon This particular unit carries a virus complex which cannot be inoculated to any potato or to Datura, and only with difficulty to tobacco, grafted to Datura it produces a very characteristic reaction only met before in the case of para-crinkle All the characters suggest a resemblance to para-crinkle On the other hand, when this Di Vernon complex is grafted to President it produces a top necrosis streak, and when to Arran Victory at best a very mild crinkle, these latter reactions distinguish it sharply from para-crinkle. On tobacco its action was curious, in general, inoculation gave no result but a small percentage developed pure and distinct Y symptoms, there was no evidence of any X virus being present

It occurred to the writer that the non-inoculability of the complex suggested Z, which he had hypothecated but not yet discovered It was therefore determined to use the Di Vernon as if it were a pure source of Z Grafts of Di Vernon, incidentally a difficult variety to graft, were employed, and the Y introduced either by graft or inoculation

Pot No. 146/231/A/31 —Both virus sources were introduced to the Arran Victory host by graft A very pronounced crinkle was produced in under 4 weeks which had every appearance of being a para-crinkle of a severe type, figs 14 and 15, Plate 9 Scions were removed on the twenty-eighth day and grafted to Arran Victory and President respectively The scions were good in both cases, the Arran Victory developed a well-marked para-crinkle, the President nothing. So far the criteria for the recognition of a para-crinkle had been satisfied The President should, however, be a carrier, a scion taken from it on July 31 and put on to Arran Victory failed to excite any reaction in the latter It may well be that this was due to the lateness of the season, or it may be that there was some other factor at work It was found that late in the season this same President plant threw up a new stem from the tuber which showed unmistakable Y necrosis.

Another experiment of the same type was made in which both the Di Vernon and the Y were introduced by grafts on to an Arran Victory

Pot No 144/146A/A/31 —At first the Arran Victory host reacted as it would to a simple Y infection, but soon this became intensified and the plant had the appearance of a perfect para-crinkle Grafts were taken from the plant as before, that in Arran Victory produced a para-crinkle but that in President rapidly developed Y symptoms, whether it was also carrying para-crinkle remains unsolved. A pair of scions were taken a month later and put in to Arran Victory and President respectively with similar results

This second attempt was not so satisfactory as the first, inasmuch as it is clear that the Y virus—whether some of it be joined up in a para-crinkle complex or not—is both free, abundant, and active within the juices of the host It will be noted that although the Y virus rendered itself still apparent, the latent streak of the Di Vernon which produces a very severe top necrosis on President was thrown entirely out of action This fact alone is evidence that a re-assortment of the virus elements must have taken place

In the writer's mind there is but little doubt that para-crinkle has been reconstructed in these two experiments, though its isolation is incomplete

§ 32 *The Synthesis of ZX and Y* —In the field we have crinkles similar in appearance to Crinkle 'A" in President which, as far as we know, have the composition ZXY Such are the common crinkles in Myatt's Ashleaf and Irish Chieftain Only two experiments were made, the viruses being introduced as Z, X and Y by grafts Both ended in failure, inasmuch as the Y virus overran the stock plants before any interaction had manifested itself

Pot No 200/140/P – In this the Y virus took complete control of the host plant

Pot No 200/144/A —Both sources were introduced by grafts, a crinkle in the Arran Victory stock developed, whose characters resembled those consequent on a Y infection from which it differed, however, in ending up as a Leaf Drop streak, an event which does not occur in a simple Y infection of this variety

We may conclude that no success has attended these efforts to synthesis ZX and Y.

§ 33 *The Synthesis of ZY and X* —The experiments fall into two groups The one where the para-crinkle employed was actually a crinkled scion from an infected Arran Victory, the other where the para-crinkle scion was derived from the latent form as it occurs in an infected President

Group I.

Pot No 249/215 3/31 —The para-crinkle scion was grafted into an X-infected President, the only reaction was a very mild interveinal mosaic

Pot No 249/215/A —The para-crinkle was grafted, the X inoculated into a healthy Arran Victory plant The scion was never vigorous and only a mixed mottle developed

Pots Nos 249/215/P and 249/215/P_1 —The procedure in these two experiments was the same as in the last, the stock plant being a healthy President In these plants a fairly definite Crinkle "A" developed and then receded A scion removed from each grafted to a fresh President produced a crinkle in one and an interveinal mosaic in the other From this it seems possible that we have built up a crinkle of the composition ZXY′

If such is the case the next year's trials will readily decide the point, then we shall have built up artificially that section of the Crinkle "A" group which is represented by Irish Chieftain and which differs from the President Crinkle "A" which has been successfully reconstructed by containing a Y in addition to ZX

In the Group II

Using the President carriers as the source of para-crinkle, X was added to it five times, three by graft and two by inoculation In no case was the reaction more than a mild interveinal mosaic which was doubtless due to the free action of the X virus

§ 34 A further experiment consisted in uniting the virus present in the Di Vernon streak carrier—which for reasons detailed in § 31 was considered a likely source of the Z virus—with the X virus This streak carrier produces a severe mosaic in Arran Victory and a top-necrosis streak in President The union of the two was brought about by grafting the Di Vernon and inoculating the X into a healthy Arran Victory, pot No 146/167/A/31 The result was a perfectly good Crinkle "A" On a President basis the result was death from top-necrosis streak A graft from the crinkled 146/167/A plant to President produced no streak but a faint mosaic. This experiment emphasises the importance of the plant's internal environment, in the President host the Di Vernon complex and the X virus appear not to have effected any reconstruction, but the former has gone its own way and killed the plant In the Arran Victory, on the other hand, something quite definite has happened: the typical clinical picture of Crinkle "A" has been produced, whilst the virus content of the plant has been so far modified that when a sub-graft was made on President under conditions fully favourable to the normal Di Vernon streak reaction, nothing happened barring a transient mild mosaic

It is of course impossible to state in terms of Z, X, and Y, exactly what has taken place, but the experiment has been recorded here because it throws light on the manner in which viruses form new complexes, whose behaviour may be something very distinct from that of the constituent elements or sub-groups which entered into the recombination

§ 35 In the efforts to build up a complex ZXY, attempts have been described involving the combination ZX, Y and ZY, X The third and far the most promising combination XY, Z has not been mentioned This is due to the fact that owing to an oversight the supposed source of XY which was used was not in reality such, but only X Now that we have a reliable source of XY, there is good hope of effecting this synthesis successfully, using as Z the N branch of the split para-crinkle pot No 250

§ 36 *The Relation of Clinical Reaction to Virus Content* —Clinical expression as a consequence of virus infection is dependent on the variety of the plant, the nature of the virus, and the character of the environment This latter as far as possible has been kept uniform , the varieties in the main are two Arran Victory and President, with whose behaviour in general we are conversant The virus infectors have been introduced under control and with a knowledge of their characters

Since we have experience that one of these viruses, viz , the X virus, will produce clinical symptoms which vary within fairly wide limits on the same potato plant within the same season, it would serve no useful end to be too meticulous in the definition of these clinical states

Adhering to the simple classification as already defined, we may tabulate (p 212) the relation between clinical expression and virus content The streak-producing viruses still await adequate analysis We know that the Di Vernon unit 4A contains Y and almost certainly Z, and that the Di Vernon unit 1A and the Uptodate unit 7A contain certainly X and Y, but whether Z or any other virus element is present in addition is unknown

§ 37 *The Creation of Virus Carriers* —By a carrier is meant a plant which contains virus elements or complexes within it which, whilst exerting no visible reaction on the plant itself, will, when grafted into another and often intimately related variety, produce an acute reaction sometimes resulting in death. Strictly, not all carriers are entirely devoid of visible symptoms throughout their growing period. Some, such as King Edward and President,

Clinical symptoms	Arran Victory	President.
Mosaic—		
Mild, veinal and mixed	Z X	
	Di Vernon 1A = ? Z, X Y	
	Di Vernon 4A = Z Y	
Interveinal	Z XY	X XY Z
Crinkle	Y ZX ZY' ZXY	ZX. ZXY
Streak—		
Leaf drop type	–	Y
Top-necrosis type	–	Di Vernon 4A = Z Y
	—	Di Vernon 1A = ? Z X Y
	Uptodate = ? Z X Y	Uptodate 7A = ? Z X Y

when carrying para-crinkle under normal conditions present, almost invariably, a picture of perfect and luxuriant health Others, such as Uptodate and Duke of York, which so commonly harbour a streak lethal to many other varieties, frequently display a mosaic often fleeting and always mild The writer has worked with two different units of Di Vernon, the one unit 1A known to contain both the X and the Y viruses and most probably the Z virus also; the second, unit 4A, contains the Y virus and the Z virus in certain but as yet undetermined combinations, but there is no evidence of any X being present The two complexes may be differentiated by their reactions on different potato varieties and other solanaceæ The plants of either of these units during one and the same season may be free from any appearance of disease or may from time to time present a mild veinal mosaic. We are ignorant as to what conditions of the environment, either external or internal, determine these variations in appearance In later sections (43 *et seq*) evidence will be given for the view that the actual virus complex itself may vary in some cases, but whether that explains the slight changes noticeable in the case of Uptodate, Di Vernon, and other known carriers, must be left at present unanswered At the moment we have insufficient criteria with which to distinguish these cases from the more completely stable class of carriers

It is scarcely necessary to point out that the phenomenon of the carrier is an essentially different one to that of resistance to virus infection, the occurrence of which in the potato is still unproved In other plants, such as cucumber, tomato and some nicotians, such resistance has been shown by several authors to have a genetic, *i e*, a chromosomic basis The "carrier" property is more probably a character of the cytoplasm As far as the writer's observations go, the passage of a complex through the body of a carrier plant has no influence on its subsequent virulence when returned to a susceptible plant, which would support the view that it is the constitution of the host's juices rather than their action on the virus complex which determines the condition

§ 38. In the course of the writer's attempts both to reproduce the clinical diseases by synthesising the unit virus elements, as well as in those to bring together within one plant two distinct and recognised diseases, a few cases have been met where the result of such unions has been to leave the recipient host in apparently as healthy a state as it was before It is true that, as in the case of Uptodate and Di Vernon, a transient and mild reaction may supervene, so it has been in these experimental cases, but if we may regard the former as true carriers, it would seem pedantic not to regard these artificial combinations as such too What they are carrying we know more or less exactly in the terms of Z, X and Y, but how they are combined and what their effects on other varieties are, remains yet to be worked out Only those cases have been included in the list of artificially induced carriers in which there is every reason to believe the constituent viruses entered, namely those in which the grafts grew freely and the inoculations were made with virulent juices over an adequate area of leaf surface

Artificial carriers arose from the following recombinations —

§ 39. *Constituent Viruses ZX + X*.—In this group there were three cases already referred to in § 28 In two there was a fleeting mild mosaic, in the third nothing, yet ZX if acting alone should react with a crinkle, and X with an interveinal mosaic

§ 40 *Constituent Viruses ZY′ (Para-crinkle in President) and X.*—In an experiment referred to in § 33, viz, Pot No 485/171/31, a President already infected with X was grafted with a President carrying para-crinkle (that it was doing so was proved at the same time by grafting a scion from it to healthy Arran Victory) The scion grew, but the effect of the union was to leave the very mild interveinal mosaic already present in the X plant in *statu quo* After an interval of 7 weeks, a scion was removed and put to a healthy Arran Victory plant; the scion itself grew from 2 inches to over 12 inches before the end of the season, yet the host plant displayed no sign of para-crinkle but only a very fleeting and mild interveinal mosaic which was scarcely noticeable In this case we have in some unexplained manner converted the highly active virus complex of para-crinkle into a non-virulent form in the body of its most susceptible victim by the addition of a further virus element.

§ 41. *Constituent Viruses ZY′ (Para-crinkle in Arran Victory) and Z Y.. (Di Vernon unit 4A, Streak Carrier).*—Pot No 146/138/A/31. In this case both complexes were brought together on Arran Victory, which reacts normally with a severe crinkle to the para-crinkle virus complex, and with a variable rather blotchy veinal mottle to the Di Vernon The grafts were made on May 29, which is perhaps the very best time of the year for such work, and grew well The host plant after showing a slight clearing of veins remained healthy to the end of the season

§ 42 It was at one time thought that the presence in one plant of Crinkle "A" and para-crinkle resulted in a condition in which neither exerted their morbid influence In one case this actually was so, but the Crinkle "A" scion, although it grew and lived throughout the season, was at no time vigorous In two other examples in which, like the preceding, the Crinkle "A" scion was grafted into Presidents carrying para-crinkle, the resultant effect was not a suppression of symptoms but a very real delay in the appearance of any reaction The incubation period of a virus complex, other things being equal, is, we know, dependent on the constitution of the complex The further relation between the nature of the symptoms suppressed and the period of incubation required for their exposition might be the decisive factor in the production of a potato virus carrier in the first season of its infection

§ 43 *Virus Complex Disintegration- Auto-infection*—In § 14 a plant has been described where different sprouts from an infected tuber displayed different clinical signs and contained different virus elements Two similar plants were observed but not studied If now a like event were to happen in the case of a plant which carried—in a latent condition—a complex built up of two or more viruses either of which when alone might be capable of producing lesions on its host, it would not, under such conditions, be surprising were either a part or the whole of such a plant, when grown on from an infected tuber, to fall a victim to one or other of the constituents of the complex it had hitherto carried

Such an event has been observed by the writer A stock of King Edward carrying para-crinkle under close observation since 1927 retained its healthy appearance throughout The stock in 1929 was all derived from an original tuber introduced in 1926 In 1930 a large batch of this material had been raised in the spring and had behaved normally , some tubers, however, were retained under insect-free conditions through the summer till July 28, when 13 tubers furnished with a second crop of long sprouts were potted up Six of these plants exhibited a peculiar very finely reticulated crinkle of a type which had never been seen before , the remainder varied from a suspicion of a mottle to a more or less severe mosaic Some of these crinkle plants were grown on by tuber in 1931 and reproduced the same symptoms, whilst others developed plants which were practically healthy in appearance. In 1931 the crinkled Edward was found by testing on tobacco to contain the Y virus in a free state throughout its tissues On President it produced a mild crinkle suggestive of the Y infection, but no necroses ensued as would be expected with a pure Y infection Grafted to Arran Victory the crinkle produced was less pronounced than that of an ordinary para-crinkle These reactions support the view that the Y′ of para-crinkle may differ somewhat from the normal Y.

If the Y virus be inoculated to a symptomless King Edward plant of the original stock the latter rapidly develops a fine veinal mottle, passing to a crinkle identical with that which appeared spontaneously in the plants under discussion

There is no reason to think that this phenomenon is due to accidental inoculation Not only is the store fumigated the whole year through twice weekly, but of the tens of thousands of tubers which have been stored therein during the last 4 years and from which plants have been raised, not one has ever aroused a suggestion of extraneous infection

§ 44 It is suggested that the para-crinkle complex in these King Edward plants has broken down and that this breaking up of the virus complex in the Edward carrier might very well have followed the secondary growth of long sprouts Indeed, Valleau and Johnson (1930) have recorded a very similar phenomenon in the Irish Cobbler, where a different virus content was observed in the tips of a long and etiolated shoot from that in the proximal portion

It is extremely difficult to prove that a phenomenon such as auto-infection occurs in the field The writer has found protection by cages and the like in the open to be eminently unsatisfactory, and the exclusion of seasonal infection by observations, however early, can never be absolute It is rather on a balance of considerations that a decision—and that not a conclusive one—can be reached The following facts suggest that auto-infection in King Edward may not be infrequent In 1930 two small field plots were grown in isolation from glasshouse tubers each belonging to a different stock from that referred to in § 43, and each one selected in 1926 and kept under close observation since then.

These two plots of King Edward, together with plots of Abundance, Arran Comrade, Arran Chief, Arran Victory, Great Scot, Kerr's Pink, Majestic and Sharpe's Express, were all planted at a minimum distance of 100 yards from each other and any other potato both in 1930 and 1931

On July 2, 1931, there were 8 out of 178 King Edward plants, or 4·5 per cent, showing the peculiar crinkle observed in the plant discussed in § 43 Two of these were tested on to tobaccos and gave the clearing of veins peculiar to the Y virus On Datura the results were negative and there was no evidence of the X virus On President the scions were poor and the reactions were not typical, no necroses occurring On Arran Victory a rather feeble para-crinkle was produced On the other hand, on Epicure a fairly typical Y leaf drop streak occurred Of the 860 plants of the other 8 varieties, all of which were derived from field plots grown in 1930 under precisely the same conditions as the King Edward of that year, there were at the same date but 10, *i e*, 1 1 per cent showing any trace of any kind of virus disease Inasmuch as all the plants from which seed was derived appeared healthy in the 1930 plots and all were exposed to the same danger of infection in that year, it is difficult to believe that some new factor has not intervened to bring about an increase of four times in the incidence of an infection normally conveyed by the green fly, in one variety only out of nine

At the end of July, 1931, a few seasonal infections made their appearance in the other plots, especially the Kerr's Pink, but none were seen in the King Edward plots

It may be urged that the sick King Edward plants were the sequelæ of late infection in 1930, but if such had been the case one would have expected a still heavier incidence and an evidence of similar infection in other plots This latter was noticeably lacking The occurrence of a few Y infected plants in the King Edward plot, together with the almost complete absence of such in the Arran Victory, Epicure, Abundance, Arran Banner and Majestic plots, all of which react readily to Y, suggests that there was some special factor responsible in the case of King Edward, and the writer suggests that this factor is auto-infection following the break up of the para-crinkle complex

§ 45 How often such cases of auto-infection occur in the field in other varieties it is impossible to say, but they may not be so rare Thus, the writer has collected in the field in Ireland tubers from apparently perfect plants of Irish Chieftain which, gathered in June, have been brought home and grown in the following season under glass Nine such were tested by grafts on healthy Arran Victory and healthy President, as well as by inoculation to Datura and tobacco One Irish Chieftain plant developed a violent crinkle, whilst the remaining eight showed slight symptoms of virus disease, mostly a clearing of the veins which in several cases disappeared The reaction of these on healthy Arran Victory and President varied from severe crinkle to a clearing of the veins and differed considerably not only in each unit but also as between Arran Victory and President in each case. A further batch of seven Irish Chieftain tubers received from Professor Murphy as virus-free and derived from plants hitherto appearing as healthy in his glasshouses were grown on by the writer in the following season under insect-proof conditions Three developed a severe crinkle, two a mosaic, and two more were highly suspicious One which developed mosaic appeared healthy in the following year and gave no reaction either on Arran Victory or President The remainder were discarded

It is obvious that some of these apparently healthy plants were in reality carriers, and that their virus content, that is, the complex or complexes of viruses they harboured, was subject to a not infrequent rearrangement

A similar state of flux as regards the virus complex carried has been observed in the variety Golden Wonder, more especially in the selected stocks of this variety lately put on the market.

Both of these varieties offer ready material for the study of the phenomenon, be it one of balance or of unstable equilibrium between the virus content and the environment, internal and external, of the carrying plant It is the latter of these two conditions which it is suggested leads to a breaking up of complexes and consequent auto-infection of the carrier plant by one of its constituent viruses.

Discussion

We may accept it as proved that in the group of mosaic diseases of the potato, one or more of the three viruses X, Y, Z occur, whether there are still others must be left open till evidence of their existence is adduced, and further that certain definite clinical diseases of the potato are due to the presence of two or more of these elemental viruses within the plant at the same moment. Such a grouping of virus elements is spoken of as a complex What then is the nature of the relation existing between the elements of a complex within the tissue juices of the potato plant ?

It is clear that whatever this relation may be in the potato plant itself, it by no means necessarily remains the same when the identical virus complex is transferred to an alien environment such as that of the tobacco or Datura. Kenneth Smith (1931, *b*) has shown how a mixture of X and Y introduced into tobacco will separate out within that plant, and that each virus can, at an appropriate moment, be recovered in a state of purity. McKinney (1931) shows the same is possible in respect to the yellow and green mosaics of wheat. Storey (1930) has shown a similar dissociation and consequent separation of a mixture of maize and cane sugar mosaic viruses within the maize The writer has shown how passage of the para-crinkle complex through a Datura with leaves will break down the complex and remove one of its constituents, namely, Y

It must be realised that whilst in the tobacco plant, for example, it is probable that the X or Y viruses coexist as a mere mixture, there seems every reason to doubt that the same holds good when the same virus elements coexist in the potato We have in the case of President plants containing the reduced crinkles of the Myatt's Ashleaf and Irish Chieftain group, examples where the X and Y viruses are present together and produce a variable but generally mild interveinal mosaic very similar to that induced by the X virus alone Now as this variety is peculiarly susceptible to a severe necrotic disease in the presence of a pure infection of the Y virus, it is difficult to regard the reaction actually induced when both X and Y are present as due to a mere free mixture or balance of the two viruses It appears to the writer that some form of combination has taken place between them, some type of linkage is set up between the two elements, which results in a reaction which is essentially that of XY and not X + Y In support of this contention is the case of para-crinkle in which Z and Y′ are both present, scores of tobaccos have been inoculated with this complex, but never have we obtained a Y reaction It is only when the aphis is brought into action that the Y element is extracted from it The supposition seems unavoidable that the Z and the Y′ are linked together within the potato in some relatively stable grouping A further reason for adopting the view that the complexes which have been under consideration are built up by the linkage of the constituent elements and are not a free and unassociated mixture of the same is the fact that, apart from the exceptional cases referred to in § 43, independent action of a constituent virus within the host is not observed As the virus element Y is so common a constituent of the complexes studied, and its presence when free in the potato so readily demonstrated in the test plant President, it is anomalous on the theory of a free mixture that we do not find occasional buds or shoots of an XY plant displaying pure Y symptoms, yet such has not been so far observed

The combination of Z and X and ZX and Y in the two types of Crinkle " A " are equally cases in point Z alone on President has no action, X on the same variety produces a mild mosaic, but ZX induces the quite distinct train of symptoms we call Crinkle " A," whilst the further addition of the Y element to it makes very little difference to the clinical reaction

A peculiar feature of virus behaviour is that when a plant is carrying a complex containing Y, a further infection of Y will nevertheless generally produce its specific reaction The following are examples of this type of behaviour —

In the case of para-crinkle carried by King Edward, an infection with Y produced a definite reaction in the King Edward, although Y′ was already present in the complex it was carrying

In the case of the Di Vernon, its complex which contained Y was transferred by graft to a healthy plant and Y introduced simultaneously , here, as we have seen in § 31, we had reason to believe that a new complex, namely, para-crinkle, was created, although there was evidence that a certain amount of Y was free in the tissues

In 1931 there was growing on the Cambridge University farm a crop of Uptodate whose general health was excellent Several plants were examined for any possible virus content and such were found to contain the Y virus and in one case the X in addition At one corner of the field the writer's President stocks infected with Y were growing, and towards the end of the season a deep fringe of diseased Uptodate plants showing the Y symptoms was seen radiating out from this same focus Such plants were presumably suffering from an added seasonal infection of a virus which they themselves already carried

It might be argued that such results as these are due to mixtures, and the final effect is the resultant balance between the actions of the coexistent virus elements

Such a view is, however, difficult to maintain because we know that virus elements such as X and Y can multiply within the plant indefinitely and the initial quantity of each constituent introduced would be unlikely to be a controlling factor It would seem more reasonable that these cases are to be interpreted as being due to the fact that the complex, say, of para-crinkle ZY′ is sufficiently firmly knit together to prevent it readily linking up with any further element such as the free Y that has been added, but that both the complex and the Y in such a case exert their respective influences independently.

The relation of the Z virus in some of the complexes such as that of ZX and ZXY, seems to be different in respect to the X and Y elements than is the relation of these two to each other Simple inoculation as opposed to grafting will cause the Z virus to be readily separated off, whilst still leaving X and Y combined. The ties by which it is held are apparently less strong than those linking the latter On the other hand, in both para-crinkle and one of the Di Vernon streak complexes, it is more securely bound to its partners, for neither of these complexes will pass either as a whole, nor do they dissociate by mere inoculation to potatoes

The occasional splitting of the para-crinkle complex (§ 14), on the other hand, shows that the complex is under certain conditions capable of spontaneous disruption It is to be noted that such dissociation takes place at points of great meristematic activity, namely, the buds, and may indeed be correlated to the phenomena of somatic mutation which, as Asseyeva (1927) and the writer (1930, *c*) have shown, are particularly common in the buds of the potato tuber A certain type of such mutations has been proved by both authors to be due to the chimaeric character of many of our domestic potato varieties where the presence of two genetically distinct tissues coexistent in one tuber can be readily demonstrated If we are right in assuming that the equilibrium of a complex is inherently bound up with the cytoplasmic environment in which it occurs—a change in that environment due to the rearrangement of the tissues might, not unreasonably, be regarded as a factor in the dissociation of a complex which has primarily existed in either or both of them

The peculiar action described in § 38 *et seq*, where an additional virus may occasionally hold up the action of an existing complex, suggests that in such cases a new linkage system is forged, in consequence of which an inactive complex results

If then it be true that the virus elements can and often do combine to form definite groups within the potato cytoplasm, it is not impossible that their quantative relation may not be a simple one, *i e*, it need not be merely a linkage of a Z, an X, or a Y element one to the other, but it is easy to conceive of groups where *n* elements of one unite with *n′* elements of another. Such a view is doubtless speculative, but there is one piece of evidence which suggests such a possibility When the virus Y was added to the Di Vernon complex, a new complex, para-crinkle, appeared Now both the Di Vernon and para-crinkle contain Z and Y What then is the distinction between them? Leaving aside the possibility that there may be an undiscovered virus element which differentiates them, it is not impossible that the difference is quantative and that if the Di Vernon complex has a constitution which we may write as ZY^n, para-crinkle might have a constitution which would be represented as ZY^{n+1} If the evidence is weak, and assuredly it is so, it would not be wise to exclude the consideration of such a view altogether.

The writer would guard against being understood to imply that he regards these linkages as equivalent to chemical valencies, rather would he compare them to the affinities which link differently endowed cellular units into one organic structure Or, to take a familiar simile, a mere mixture of viruses might be compared to three strangers present in a street, each going about his own business in his own way—one loitering about the shops, another heading in one direction, a third running in the other The type of linkage which it is suggested is at work in a virus complex is one that would be represented by these same individuals marching arm in arm in one definite direction at a definite pace and with a definite purpose Such a trio is for the time a complex, but not necessarily permanent, rather is it one which would readily disrupt if translated to a strange environment or exposed to an unexpected encounter One need not press the simile, it is the difference between an organised and unorganised collection of units

Whether viruses are to be regarded as living organisms or as non-living enzyme-like bodies, is beyond the scope of this discussion The suggestions put forward, however, are consistent with the view that the viruses may be living organisms which have acquired, perhaps by the very reason of their extremely minute size, special relationships between themselves and their environment which allow of the building up of groups or complexes capable of existing and multiplying as such within a given environment

Throughout the last 18 months I have received abundant assistance in every respect from Mr F C Bawden, to whom I most gladly tender my best thanks

Summary

Following on the isolation of the X and Y viruses by Dr Kenneth Smith, the writer has discovered a third virus element which he has named the Z virus. A table is given of the reaction on different potato varieties of each of these three viruses acting alone under glasshouse conditions

The Z virus acting alone has a very limited pathogenicity, but acting in unison with either X or Y, or both, it brings about the diseases we know as Crinkle "A" and para-crinkle It is also present in a streak-carrying Di Vernon and probably in many other infected plants

As a result of both analytical and synthetic methods it has been possible to ascribe compositions in terms of X, Y and Z, to certain definite clinical virus diseases.

When certain of the virus elements X, Y or Z, are brought together experimentally in appropriate grouping, it has proved possible to build up such clinical pictures as Interveinal Mosaic, Crinkle "A," and Para-crinkle, *i e*, virus complexes have been synthesised in the potato.

Reason is given for the suggestion that the Y′ of para-crinkle differs somewhat from the common Y of the other potato diseases There is not at present enough evidence to claim for it a substantive position as a distinct virus element

The reaction of a virus complex is not the mere summation of that of its two constituents A virus complex is not a mere mixture—it is a linked group

The view is put forward that the grouping of the virus elements in a complex is not to be regarded as comparable to a chemical valency, but rather to a linkage whose strength varies with the nature of the viruses concerned and in particular with the internal environment of the plant

Evidence is given showing that the addition of a further virus element, or of a complex of such, to a pre-existing virus complex may, in the first season at least, bring about a condition in which no clinical reaction occurs and the plant behaves as a carrier

A virus complex when present in a potato plant and exerting on it a definite reaction, may at some active growing point in the plant spontaneously break up and allow of one or other of its constituent elements to pass alone into a growing bud and there express itself This phenomenon has been observed on three occasions in para-crinkle in Arran Victory

If a similar spontaneous splitting takes place in a carrier plant, one of the free elements may exert a pathological effect in its own capacity and convert the symptomless carrier into an openly sick plant Such a phenomenon is described as one of auto-infection It has been definitely observed in one variety and is suspected in two others

Inclusions or X-bodies are found to be present in infections produced by the X virus, or where the X virus is present, to be absent in those consequent on the Y virus alone and, with a doubtful exception, absent in that due to the Z virus alone

DESCRIPTION OF PLATES

Plate 6

Fig 1 —Pot 168/31 A leaf of President infected with Crinkle "A" as a result of grafting from a similar plant The bright mottling, waving and deformity are the common characters of crinkle

Fig 2 —Pot 167/31 Arran Victory infected with Crinkle "A" as a result of inoculation, producing a rather diffuse, mild mosaic with no deformity or sign of crinkle

Fig. 3 —Pot 250/P5/29 The top of a President plant showing an infection by graft with Crinkle "A" in the current season This crinkle contains the viruses X and Z The signs of disease are similar to those seen in fig 1. The brilliance of the mottling is not so great as that shown in fig. 4

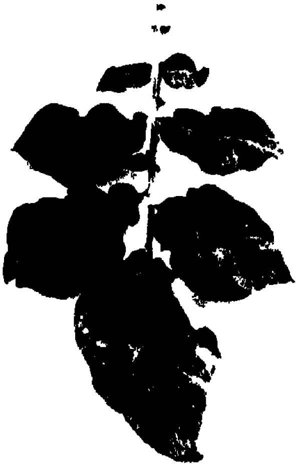


Fig 1

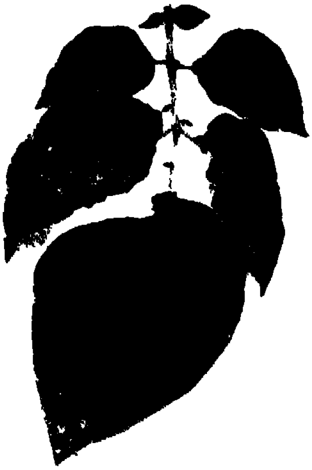


Fig 2

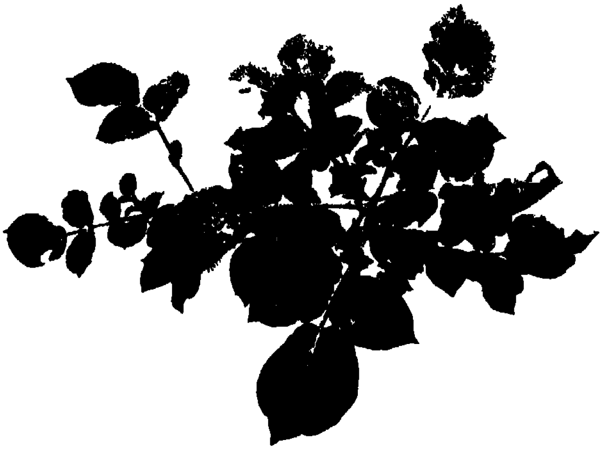
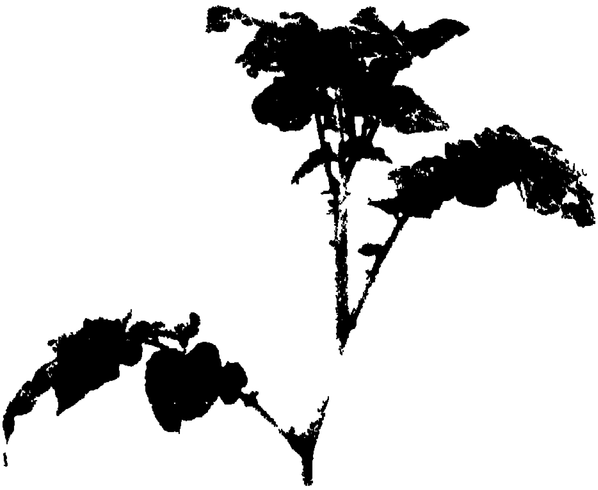


Fig 3



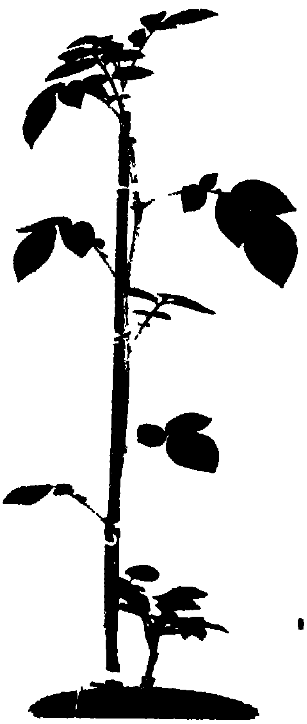
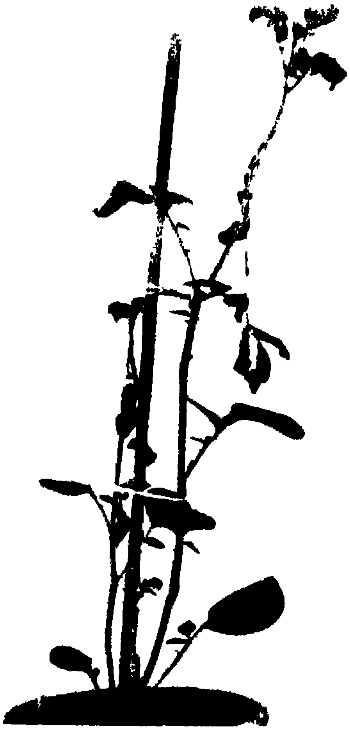


FIG 5



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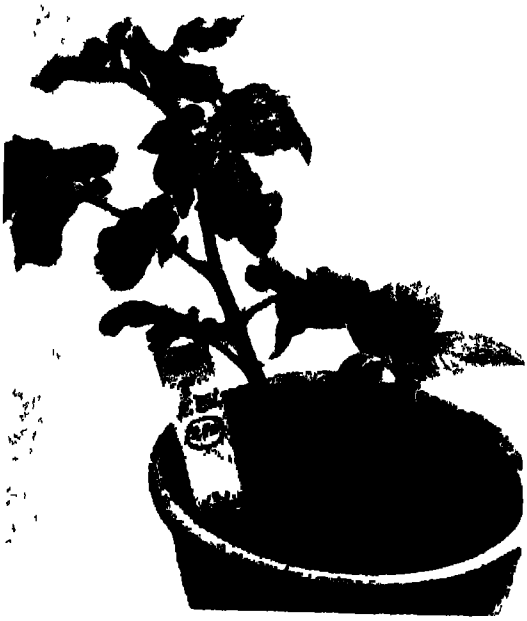

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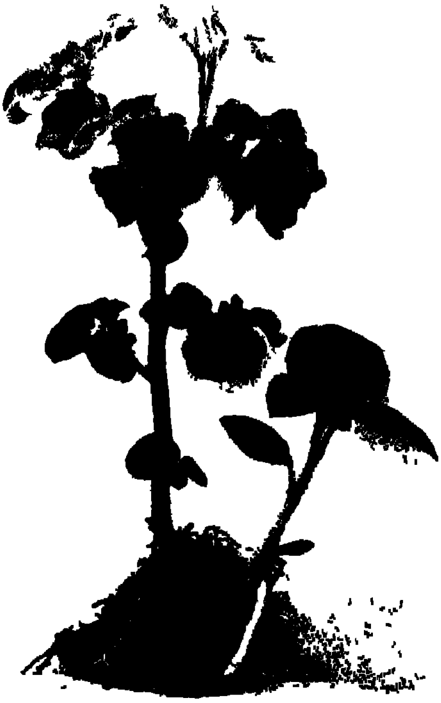

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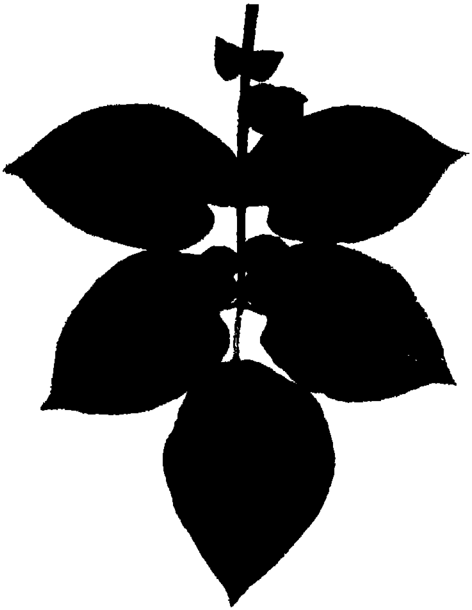

Fig 9

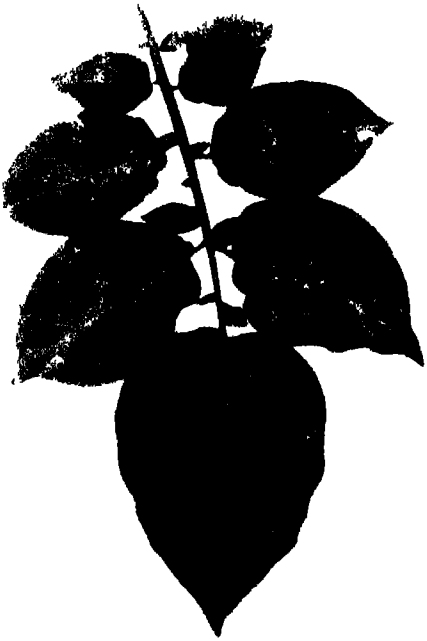

Fig 10

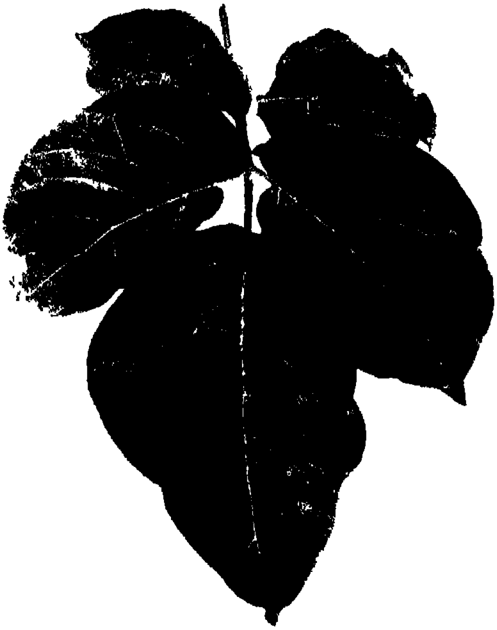

Fig 11

Fig 12

Fig 13

Fig 14

Fig 15

FIG 4—Pot 730/29 The top of a President plant showing an infection by graft with Crinkle "A" derived from Myatt's Ashleaf and containing the virus element X, Y and ? Z The signs of disease are similar to those seen in fig 1, but the brilliance of the mottling is considerably greater

PLATE 7

FIG 5—Pot 144/Dat P/31 A healthy President plant on which has been grafted a leaved Datura scion on which is a scion infected with the Y virus This latter scion is partly obscured in the photo by the Datura leaves. The President remained perfectly healthy throughout the season

FIG 6—Pot 144/Dat P1/31 A healthy President plant has been grafted with a leafless Datura stem on which has been grafted a Y-infected scion The President is dying from leaf drop streak as a result

FIG 7.—Pot 250/31 An Arran Victory plant infected in 1928 with para-crinkle in which the virus complex "split" in 1931, giving rise to one diseased shoot D and one quasi-healthy one N On the latter shoot it is to be observed that the veins are cleared, it contains the Z virus

FIG. 8—The same plant as in fig 7, but removed from its pot and washed so as to show the relation of the shoots D and N to the mother tuber

PLATE 8

FIG 9—Pot 221/29 A leaf from an Arran Victory plant which has formed the basal stock of a double Datura graft with leaves, with para-crinkle as the infecting scion The mosaic mottle produced is due to the virus Z, which is the same as that present in the N shoot shown in figs 7 and 8

FIG 10—Pot 250/A3/29 An intermediate leaf of an Arran Victory plant infected by grafting with Crinkle "A" The mottling is mainly veinal, waving and deformity is slight, though often more marked than shown here

FIG 11—Pot 249/AA2 A lower leaf of an Arran Victory plant infected by grafting with Crinkle "A" in the latter part of the season. The mottling is now very diffuse, the surface smooth and characteristically glazed

PLATE 9

FIG 12.—Pot 559/K Ed Dat A1/30 An Arran Victory plant in which a Crinkle "A" has been synthesised. Note the similarity of the lesion in the upper part of plant with that shown in fig 10, and in the lower part with that in fig 11

FIG 13—Pot 559/K Ed Dat P1/30 A leaf from a President plant in which Crinkle "A" has been synthesised Compare with fig 1

FIG 14—Pot 146A/231A/31 A leaf from an Arran Victory plant in which para-crinkle has been synthesised The veinal mottling, the extreme waving and deformity, are of the same intensity and character as those in normally infected Arran Victory plants. Compare fig. 15

FIG. 15—Pot 138/31. The leaf of an Arran Victory plant in its third season of infection with para-crinkle.

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The Effect of Spaced Radiation on Tissue Cultures in vitro

By F G Spear (from the Strangeways Research Laboratory, Cambridge)

(Communicated by Sir Frederick Andrewes, F R S —Received December 16, 1931)

I *Effect on Mitosis*

In a paper by Canti and Spear (1929) it was shown that exposure of tissue cultures to 100 mg radium* (element) for 2½ minutes at 0 5 cm (platinum filter 0 5 mm) produced a fall in mitosis followed by a compensatory increase, after which mitosis became normal, fig 1 The same paper described the variation in mitotic activity at different periods after exposure to the same radium for 9 minutes From the data given it is possible by interpolation, to construct a "mitosis curve" for the 6 hours following an exposure of 5 minutes' duration, fig 2

The following experiments were made in order to study the effect of two irradiations of 2½ minutes each, separated by two different time periods

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* The radium was kept in a circular container 13 mm in diameter and 1 mm deep (internal measurements)

Object of Experiments —To compare the effect on mitosis *in vitro* of exposure to a continuous irradiation with the effect following exposure to a spaced irradiation of the same intensity

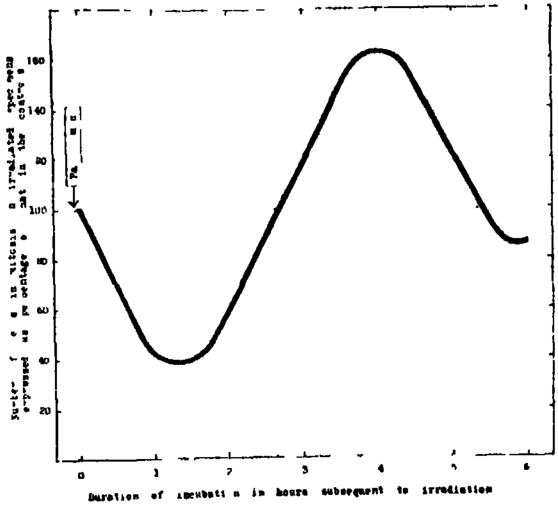

Fig 1 —Curve showing the variation in mitosis during the first 6 hours subsequent to an irradiation of 2½ minutes

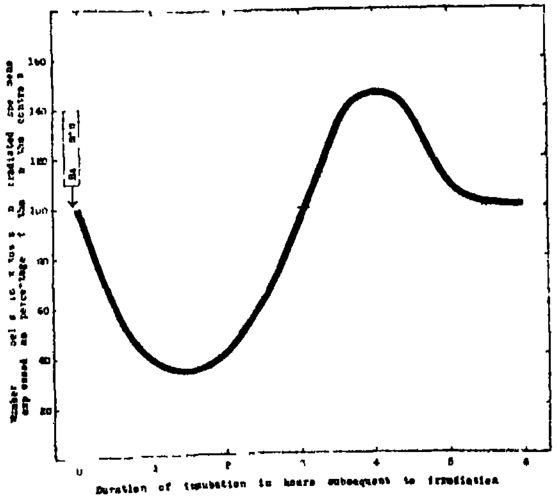

Fig 2 —Curve showing the variation in mitosis during the first 6 hours subsequent to an irradiation of 5 minutes

Method—The material used was derived from the choroid and sclerotic of fowl embryos and was grown in fowl plasma and embryo extract by the hanging drop method

Growths of the second subculture were used Exposures to irradiation were made in the special apparatus described in a previous paper (Canti and Spear, 1927) In the present experiments comparable sets of cultures were selected , one was kept as control and the other was exposed to 100 mg Ra (Pt filter 0 5 mm) at 0 5 cm for 2½ minutes and returned to the incubator After an interval of 2⅔ hours following this first irradiation, the experimental cultures were exposed to a second irradiation of the same duration and at the same distance as before The cultures were fixed and stained 80 minutes after this second irradiation Other sets were given a similar double irradiation and were incubated for 2⅔ and 4 hours respectively, subsequent to the second irradiation They were then fixed, stained and compared with their corresponding controls

A second series of cultures was irradiated with an interval of 80 minutes between the two exposures One set of specimens was incubated for 80 minutes, and another for 5½ hours, following the second irradiation, and after incubation, were fixed, stained, and compared with their corresponding controls

Results —The experimental conditions and the results are summarised in Tables I and II and are plotted as curves in figs 3 and 4

Table I

(Figures in italics refer to controls, those in roman type to irradiated specimens)

	Period of incubation after first radiation						Period of incubation after second radiation.					
	Immediate examination		80 minutes		2½ hours		80 minutes		2⅔ hours		4 hours	
Number of cells in mitosis	*21* *30* *22* *21*	22 27 22 26	*28* *31* *24* *20*	11 12 11 6	*38* *34* *35* *42*	41 31 25 26	*63* *40* *39* *51* *35* *64*	30 14 18 20 20 18	*21* *22* *32* *36* *21* *22*	17 13 16 28 20 13	*34* *43* *20* *22* *38* —	28 57 25 39 36 —
Totals	*94*	97	*103*	40	*149*	123	*292*	120	*154*	107	*157*	185
Average per slide	*23 5*	24 3	*25 8*	10	*37 7*	30 8	*48 7*	20	*25 7*	17 8	*31 4*	37
Per cent	103 4		38 8		82 6		41 1		69 3		117 8	
	These figures are taken from the earlier paper by Canti and Spear (1929)						Cultures were irradiated for 2½ minutes, returned to incubator for 2⅔ hours, then given a second exposure to radium for 2½ minutes and examined after period indicated.					

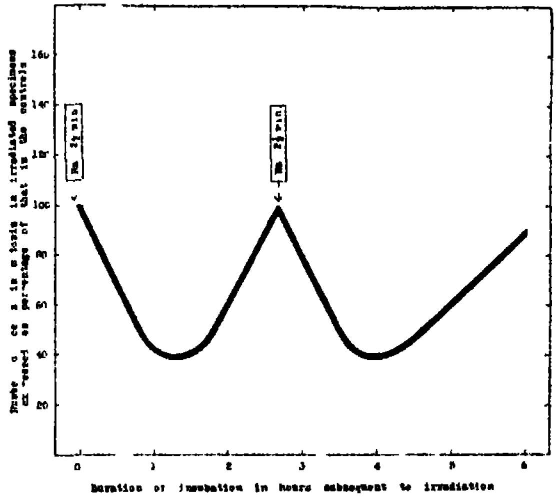

FIG 3 —Curve showing the variation in mitosis after each of two irradiations of 2½ minutes The curve is drawn from the figures given in Table I

Table II

(Figures in italics refer to control cultures, those in roman type to the irradiated specimens)

	Period of incubation after first radiation				Period of incubation after second radiation			
	35 minutes		80 minutes		80 minutes		5½ hours	
Number of cells in mitosis	*21* *30* *22* *21*	17 14 22 14	*28* *31* *24* *20*	11 12 11 6	*63* *40* *39* *51* *35* *64*	11 19 17 16 25 13	*34* *43* *20* *22* *38* –	19 25 15 50 23 —
Totals	*94*	67	*103*	40	*292*	101	*157*	132
Average per slide	*23 5*	16 8	*25 8*	10	*48 7*	16 8	*31 4*	26 4
Per cent	71 5		38 8		34 6		84 1	
	These figures are taken from the earlier paper by Canti and Spear (1929)				Cultures were irradiated for 2½ minutes, returned to incubator for 80 minutes, then given a second exposure of 2½ minutes and examined after period indicated			

Discussion—Cell division is a very sensitive biological indicator of the effect of radiation and affords a useful means of comparing the effectiveness of continuous and spaced radiation when the dose administered is only just above the minimum threshold value required to produce a demonstrable

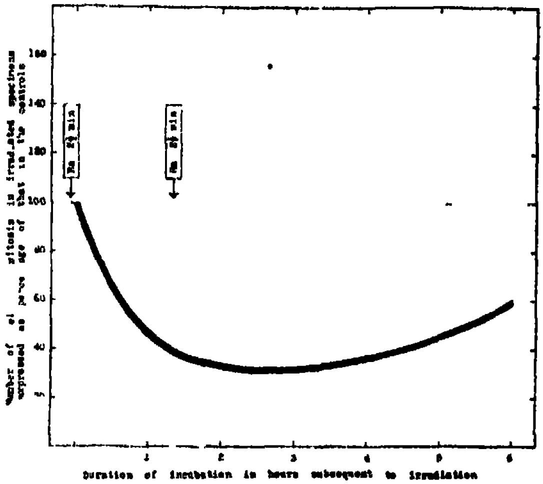

FIG 4 —Curve showing the variation in mitosis after each of two irradiations of 2½ minutes. The curve is drawn from the figures given in Table II

effect at all With doses of this order, a slight alteration in the time of administration produces a marked difference in the effects observed, but the "working margin" is so narrow that it is extremely easy to miss such variations in effect altogether The question may be of importance, however, in considering the effect of radiation on a cell (*e g*, on the periphery of a tumour undergoing irradiation) which is only just within the range of the gamma rays

The reason for the diminution in the number of cells in mitosis in tissue cultures after exposure to 100 mg radium for 2½ minutes, fig 1, has been shown to be due to the fact that while cells already in mitosis at the beginning of irradiation continue to divide in apparently normal fashion, a proportion of the other cells which would normally have divided becomes delayed in entering mitosis (Strangeways and Hopwood, 1926, Canti and Donaldson, 1926) Mitosis is at a minimum about 80 minutes after exposure to radium and thereafter the number of cells in mitosis increases and reaches normal 2⅔ hours after the time of irradiation This point marks the limit of action of the radium, no further cells are affected as a result of the exposure Also at this point those cells which were delayed now begin to divide and consequently the number of cells entering mitosis per unit of time is increased The "mitosis curve" therefore rises, reaching a maximum 80 minutes later (*i e*, 4 hours from irradiation) The only other likely possibility which could explain the rise in mitosis is that the time occupied by the process of cell division is increased and experiments now being done by cinematography on this point* show that there is no significant increase in the duration of the mitotic process after an irradiation of this order

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* Canti and Spear, unpublished work

It appears therefore that irradiation prevents a certain number of cells from entering division, but that after the removal of the radium these cells divide When specimens are fixed 4 hours after radiation has ceased, the maximum number of these cells in which mitosis has been temporarily inhibited are present in addition to the cells in division which have not been affected by the irradiation at all It would appear that few, if any, cells are actually killed or permanently prevented from entering mitosis because the compensatory increase almost exactly equals the original diminution

The results of the experiments described in this paper indicate that, when 100 mg radium are used, two irradiations of 2½ minutes each suitably spaced (*i e*, 80 minutes apart) have a much greater effect upon mitosis than that following 5 minutes' continuous exposure The effect of the spaced radiation is of the same order as one irradiation of 2½ minutes with 300 milligrams radium (Spear, 1931 (i))

A spacing of 2⅔ hours (*i e*, 80 × 2 minutes) has a less marked effect on the diminution of mitosis

The ∽-shaped form of the curve of fig 1 indicates that an irradiation of 2½ minutes (100 mg Ra) affects cells up to 2⅔ hours previous to (visible) mitosis Therefore a double irradiation in which the spacing-interval is 80 minutes hits a number of susceptible cells twice during their sensitive period Further, as described above, the first irradiation temporarily delays the onset of visible mitosis in a proportion of cells and it would seem that such delay involves a prolongation of the period of " premitotic " sensitivity Moreover, delaying the mitotic process also seems to be correlated with an increase in the radiosensitivity of a cell since a second irradiation given 2⅔ hours after the first prevents any of the " delayed " cells from entering mitosis during the period of observation. The second irradiation also affects a group of cells (which were " out of reach " of the first irradiation) in the same proportion as the original irradiation, giving a W-shaped mitotic curve That mitosis should still be below normal 5¼ hours after the second irradiation, when the spacing-interval is 80 minutes, suggests that an irradiation of 2½ minutes can produce changes in cells which are not demonstrable unless enhanced by a second irradiation given 80 minutes after the first.

A spacing-interval of about 80 minutes between the two irradiations seems to be the optimum for producing the greatest effect on mitosis Alteration in this interval, whether by increase or decrease, lessens the effectiveness of the radiation in reducing mitosis

II *Lethal Effect*

I have shown in a previous paper (Spear, 1931 (i)) that exposure of tissue cultures to 300 mg radium (element) for 2½ minutes at 0 5 cm (0 5 mm Pt filter) causes a temporary diminution in mitosis (which is minimum 80 minutes after the exposure), normal activity being regained 6 hours after exposure

It has also been shown (Spear, 1930) that tissue cultures may survive an exposure of 3 *hours* to 300 mg radium under the same conditions Four cultures out of six, however, died within 3 weeks

The following experiments were made to determine whether a short irradiation, which produced only a temporary effect on mitosis when given once, could, by repetition at intervals, cause a delayed lethal effect in the cultures irradiated It was therefore decided to give a total spaced irradiation of 60 minutes with 300 mg radium (element) acting at 0 5 cm (platinum filter 0 5 mm) and to give the irradiation in 24 separate exposures of 2½ minutes each at intervals of 80 minutes

Object of Experiment—To observe the effect on tissue cultures of short, frequently repeated irradiations

Method—Cultures were selected from material grown for 24 hours after the second subcultivation Of these 12 were used for irradiation and 12 kept as controls Exposures were made at a distance of 0 5 cm, to 300 mg radium in the special apparatus, previously referred to, placed on the laboratory bench Cultures were irradiated in pairs and during the period of exposure the corresponding pair of controls was left on the bench outside the incubator so that any chilling effects were the same for all the cultures The coverslips were resealed with paraffin wax periodically in case frequent alteration in temperature or radiation had produced any minute cracks in the wax which would cause evaporation of the culture medium

Twenty-four exposures were made, each of 2½ minutes' duration, *i e*, a total irradiation of 1 hour The cultures were returned to the incubator for 80 minutes between the irradiations

The first irradiation was made shortly after noon on one day, and the last at 8 0 p m the following day Cultures were subcultivated immediately after the final irradiation and every 48 hours thereafter so long as they survived Four were fixed for examination during the progress of the experiment.

A second batch of 24 cultures was similarly treated 2 months later

Results—The results are given in Table III, which summarises the history of each irradiated culture subsequent to exposure

Table III—Showing the History of Cultures exposed to Spaced Radiation subsequent to their removal from the Radium

Culture No.	Day																						Number of subcultivations before death	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
1	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead		10	
2	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead								7	
3	S	—	S	—	S	—	S	—	Fixed with control														—	
4	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead								7	
5	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead				9	
6	S	—	S	—	Fixed with control.																		—	Average 7 7 (excluding fixed specimens)
7	S	—	S	—	Infected and discarded.																		—	
8					Fixed with control 80 minutes after final irradiation																		—	
9	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead								7	
10	S	—	S	—	S	—	S	—	S	—	Dead												5	
11	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead				9	
12	S				Fixed with control 24 hours after final irradiation																		—	
13	S	—	S	—	S	—	S	—	S	—	S	—	Dead										6	
14	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead		10	
15	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead								7	
16	S	—	S	—	S	—	S	—	Infected and discarded														—	
17	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead						8	
18			Broken during irradiation																				—	
19	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead								7	Average 7 0
20	S	—	S	—	S	—	S	—	Dead														4	
21	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	S	—	Dead		10	
22	S	—	S	—	S	—	S	—	S	—	S	—	Infected and discarded										—	
23	S	—	S	—	S	—	S	—	S	—	S	—	Dead										6	
24	S	—	S	—	S	—	S	—	S	—	Dead												5	

All controls showed plentiful mitosis and outwandering throughout the experiment

S = subcultivation.

Average for whole series 7 3

Discussion —If these results are compared with those following continuous radiations (Spear, 1930), it is evident that the radiation effect is much enhanced by spacing the irradiation—1 hour's exposure with spaced radiation being more effective than 3 hours continuous irradiation The average survival periods following continuous exposures of different duration described in the previous paper already referred to were as follows —

Table IV —300 mg of radium filtered through 0 5 mm platinum, acting at 0 5 cm distance

Hours of exposure	Number of subcultures before death								Average
3	20*	20*	10	10	10	9	—	-	10 +
6	8	7	6	6	6	5	—	-	6
9	8	8	5	4	4	3	—	—	5
12	5	4	3	3	3	3	3	2	3
18	3	2	2	2	2	2	1	—	2
24	2	1	1	1	1	1	1	-	1
	Each figure has reference to one irradiated specimen								

* Recovery of culture

These average figures are plotted as a curve in fig 5 From the graph it is seen that a survival period of 7·3 subcultivations (i e , the figure resulting from *one* hour's spaced irradiation) corresponds to a continuous irradiation of 4½ hours

Classical work on the effect of dividing a given dose of radiation has been done by Regaud and Ferroux (Regaud and Ferroux, 1929, 1930) These workers have shown that, while it was impossible to produce sterilisation of the testicle of the rabbit by a single irradiation without causing severe damage to the overlying skin, complete sterilisation could be effected by means of a spaced irradiation which caused no ulceration of the integument Using X-rays, the optimum conditions were about 5000 R units (8 0 mm Al filter) given in five equally spaced fractions over a period of 4 to 9 days

Alberti and Politzer (1924), working with X-rays on amphibian larvæ, found that the optimum spacing when only two irradiations were given was 4 days

Edinow and Mottram (1931) have recently shown that Jensen's rat sarcoma is slightly more sensitive to spaced than to continuous (gamma) radiation of the same total duration In this work also the interval between the exposures was measured in days

I have previously shown (Spear, 1931 (II)) that, when tissue cultures are exposed to radium at 1-day intervals, it is immaterial whether a lethal irradiation is given in one dose of 6 hours or in six equal fractions of 60 minutes at 24 hourly intervals

The present work suggests, however, that, at any rate with tissue cultures, effective use may be made of quite short exposures to radium if the dose is frequently repeated at suitably short intervals which can be measured in minutes Since with such an arrangement of doses, cells are not prevented from entering mitosis, successive groups can be irradiated in the premitotic sensitive stage until the whole culture becomes degenerate

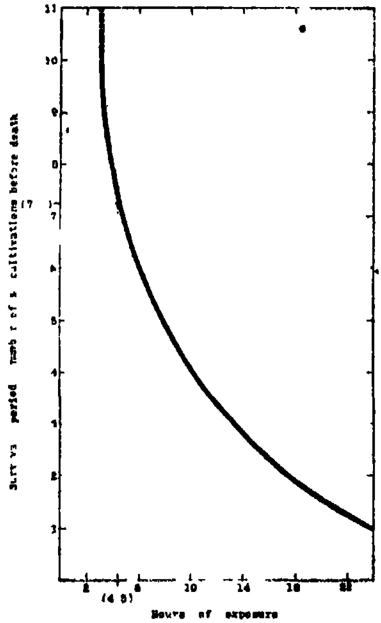

Fig 5—Curve showing the average survival periods before death of cultures exposed to continuous irradiations with 300 mg radium The curve is drawn from the figures given in Table IV

Summary of Results

(1) Two exposures of 2½ minutes each, separated by a time-interval, produced a different effect from a continuous irradiation of 5 minutes

(2) When tissue cultures were given two irradiations, each of 2½ minutes, with 100 mg Ra (element) at 0 5 cm (Pt filter 0 5 mm), diminution in mitosis was more marked when 80 minutes elapsed between the exposures than when the interval was 160 minutes

(3) Repeated exposure to 300 mg Ra (element) at 0 5 cm (Pt filter 0 5 mm) enhanced the effect of radiation It was found that 24 exposures, each of 2½ minutes, separated by 80-minute intervals (*i e*, a total exposure of 1 hour) produced a delayed lethal effect equivalent to that following a continuous irradiation of 4½ hours

Acknowledgment

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612 229 597 48 Lepidosiren

Experimental Researches on the Emission of Oxygen by the Pelvic Filaments of the Male Lepidosiren *with some Experiments on* Symbranchus marmoratus

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The stage which the question of the function of the pelvic filaments of the male *Lepidosiren* had reached before the researches described in this paper can be seen by reference to the paper by Carter and Beadle (1930) and that by Cunningham in the previous year The researches of Carter and Beadle as well as those previously carried out by Graham Kerr were made in the Gran Chaco of Paraguay, in the swamps of which region *Lepidosiren* is rather abundant But when direct experiments on the function of the filaments were contemplated the political conditions made it inadvisable to attempt to visit this region, and it was suggested that *Lepidosiren* would be found in sufficient abundance on the island of Marajó at the mouth of the River Amazon No evidence was obtained that the fish had recently been taken in that island, but three specimens, all from the same locality, namely a "papyrus meadow" near Fazenda Dunas on the north coast of the island, were recorded in 1896 and 1898 by Dr Emil A Goeldi, Director and founder of the Muséu Goeldi at Belem

It was therefore decided to organise and carry out an expedition to Marajó. The equipment was prepared in the Physiological Department of the London Hospital Medical College and consisted of large glass tubes from 18 inches to 30 inches in length and 1½ inches to 3 inches in diameter, and weighed quantities in hermetically sealed tubes or bottles of the reagents required for the estimation of dissolved oxygen in water, together with the necessary accessories, and a special pump for obtaining water from below the surface of swamp pools

We left Liverpool on November 16, 1930, and arrived at Belem (Pará) on December 3 We could obtain no definite information as to the occurrence of *Lepidosiren* in the immediate vicinity, we therefore decided to proceed to the island of Marajó

After spending nearly four weeks at a Fazenda called Camburnpy in the interior of the island, and failing to obtain any information or evidence that *Lepidosiren* occurred in the neighbourhood, we travelled by sailing boat round Cape Magoary to Fazenda Dunas, where we arrived on January 16

We obtained our first *Lepidosiren* on January 24, 8 days after our arrival at Dunas It was a female (80 cm long) and dead when brought to us The man who brought it had seen it in the water and caught it with a basket The ovaries were large and no longer fresh, but apparently the fish had not yet spawned The first male was received on February 9, was 63 cm long and had filaments about 5 mm in length, and had been brought from the east part of Fazenda Dunas In spite of the aid of Senhr Cavalcante we did not succeed in catching any specimens ourselves This first male and nearly all the others of both sexes which we obtained afterwards were brought to us in consequence of the offer of rather high prices, especially for males with filaments, and all with one or two exceptions were caught and brought to Dunas by a few men from a place called Bonito on the coast, a considerable distance to the west

For some time the pelvic filaments of all the males were only a few millimetres in length, and therefore only beginning to develop We also received a few nests taken from the nest-burrows, and the eggs which they contained were either only just fertilised or showed very early stages of development It was therefore evident that in the second week of February the breeding season had only just commenced and it was not till the third week of March that fish with well-developed filaments were obtained

On February 10 a male and female said to have been obtained from the same burrow were brought to us, and also some eggs which were taken from the nest These eggs when they reached us contained a very early embryo extending round half the circumference of the yolk It may be inferred that in this case the burrow had been opened up when a pair of the fishes were in it and actually engaged in the deposition and fertilisation of the eggs Some eggs escaped from the female while it was being handled The burrow in which the fish were taken was said to be 8 feet long The filaments of the male were only about 5 mm in length, and showed no red colour, only grey, and it may be inferred that the greater part of the growth of the filaments takes place after the eggs are fertilised

Method used for Estimation of Amount of Dissolved Oxygen in Water

We took with us a Van Slyke apparatus for measuring the volume of gases extracted from 10 c c of water in a mercury vacuum, but after a few trials found that it was not satisfactory for our purpose

The iron sulphate method described by Legendre in a paper published in 1909, was also thoroughly tested and found unsuitable

The Winkler method, by which we obtained definite and consistent results, is so well known that it is not necessary to describe it in detail Carter and Beadle (*loc cit*, p 218), who used the Winkler method exclusively, found that in the lower layers of water in a typical swamp in the Gran Chaco there was a great deficiency of oxygen, in the central part of the swamp in the dry season oxygen was entirely absent except at the surface, after heavy rain the amount rose to 0 5 c c per litre In the shallow part among the weeds the amount was usually from zero to 0 2 c c but occasionally rose to 1 c c In the open pools where the weeds were absent the amount of oxygen was never much below 1 c c per litre

It was a necessary part of our reseaches to ascertain how far similar conditions existed in the waters of the regions which we visited We obtained samples from below the surface by means of a double action Solo pump designed for spraying limewash or insecticides The pump had no air chamber and pumped water without drawing air By inserting the brass delivery tube down to the bottom of a jar or bottle and pumping till the water had overflowed for some minutes and then closing the receptacle with a rubber stopper, the water was obtained without contact with air Whenever possible the end of the suction tube was attached to a stick which was thrust into the mud at the bottom of the water which we were sampling Carter and Beadle's method of using a smaller bottle and a siphon contained in a larger jar was not suitable for obtaining quantities of about a gallon or more needed for our experiments At Camburupý we found that all the swamps were dried up. A sample of water taken from the Rio Cambu at 2 feet below the surface contained 3 68 c c oxygen per litre, but this was not stagnant water as it was open to the tide Another sample from the Igarapé Grande, at the same depth, gave 3 8 c c per litre, the temperature being 31 5° C

At Dunas a sample of water taken 2 feet below the surface, from a marsh pond or small swamp near the upper part of the Rego do Jacaré Magro, contained only 0 39 c c oxygen per litre This would have been a very suitable degree of deoxygenation for our experiments, but it was not practicable to obtain a regular supply of the water from such a distance We therefore often used well-water, reducing the oxygen content by putting a small fish (*Callichthys*) into a jar full of it, closing the jar with an air-tight rubber stopper, and leaving it till the respiration of the fish had ceased We also found that deoxygenated water could be obtained from a depth of about 18 inches from a large pool near Dunas House

Experiments and Results

The experiments on the effect of *Lepidosiren* on the dissolved oxygen were made in the long glass tubes The water was siphoned into the tube from the jar, the end of the siphon delivering the water at the bottom of the tube In the earlier experiments liquid paraffin to the depth of about 1 inch was put into the tube before the water, so that it floated on the surface of the water and excluded the air Sometimes the fish was suspended in the tube by a string passed through the skin of the lip or through the branchial cavity, but both these proceedings were found to have a bad effect on the fish. Also the paraffin was found to irritate the fish, probably by obstructing its respiration as the oil became churned up with the water, with the result that specimens so treated only survived a few days Later, therefore, the fish was simply lowered into the water tail downwards, and usually it made little movement when treated in this way. The samples of water were drawn off by a siphon from near the bottom of the tube, and it was assumed that oxygen would not diffuse far from the surface. Paraffin, however, was always used in the graduated cylinders in which the analysis was made, about an inch of paraffin being placed at the bottom of the cylinder and the water delivered beneath it. It should be noted that the fish was able to respire air at the surface of the water during the experiment, so that the blood was kept supplied with oxygen If the fish had been placed with the water in a vessel closed by an air-tight stopper, any increase in the dissolved oxygen might have been due to air expelled from the lungs

The results of our experiments are recorded in Table I (males) and Table II (females) It will be seen that in nearly all cases there was an increase of dissolved oxygen when the fish was introduced This must be attributed to dissolved oxygen in the water on the skin of the fish, and partly to the surface water carried down by the fish as it was put into the tube With males 2 to 9 it will be seen that there was usually a decrease of dissolved oxygen with time, with the exception of No 2, February 11, and No 9, March 3 With regard to the last there was evidently a decrease at the first test and at the others the increase was very slight In No 2, February 11, the first test showed a distinct increase, which was diminished in later tests In the experiments on females there was a decrease in dissolved oxygen after every interval, which could not be due to absorption by filaments, as these were entirely absent The question arises whether the decrease was due to absorption of oxygen by the skin, that is, to cutaneous respiration But in these experiments the fish, whether male or female, was able to, and did, breathe air by the mouth and lungs, therefore the oxygen tension in the blood would probably not have been as low as it was in the water surrounding the fish in the tube But apart from this there were strong indications that the decrease was due to the accumulation of mucus from the skin of the fish This mucus adhered to the hands when the fish was handled It made the water into which the fish was put look turbid, and as it was heavier than water, accumulated at the bottom of the experimental tube Its effect was seen in the graduated cylinder when the reagents were added It was not precipitated by the manganese chloride, but when the caustic potash and potassium iodide were added it was precipitated with the manganese hydrate, the precipitate having a dense opaque white appearance with very little yellow colour The precipitate was only slowly dissolved by the hydrochloric acid and these features were always associated with an apparently small amount of dissolved oxygen, as indicated by the quantity of thiosulphate used It is known that organic matter absorbs iodine and the effect of this in diminishing the quantity of oxygen estimated by the Winkler method is mentioned in text-books. It might be suggested, therefore, that the apparent diminution in the amounts of oxygen was merely due to the increase in the amount of mucus present with the lapse of time, and the corresponding absorption of iodine by the mucus On the other hand, the decrease might be caused partly by absorption of dissolved oxygen by the mucus

Table I.—Experiments on Males Winkler Method Oxygen, cubic centimetres per litre

	Number and date.	Original water.	After Lepidos.	1st test	2nd test.	3rd test	4th test	Water and remarks
A	No. 2 (February 10)	1 28	—	1 78 (5 mins)	1 17 (15 mins)	—	—	Water from a glass jar in which a small fish was living Filaments 6 mm long
B	No 2 (February 11)	—	1 49	2 06 (20 mins)	1 72 (30 mins)	1 67 (30 mins)	—	Well water, oxygen reduced by small fish.
C	No 2 (February 13)	0 91	1 73	0 91 (15 mins)	1 00 (30 mins)	—	—	Well water boiled, then oxygen reduced by small fish Much mucus
D	No 8 (February 23)	1 73	1 11	0 9 (20 mins)	—	—	—	Well water, oxygen reduced by small fish Only incipient filaments
E	No 9 (February 27)	1 73	1 90	1 56 (10 mins)	0 9 (20 mins)	—	—	Oxygen reduced by small fish *Lepidosiren* suspended by string through branchial cavity Filaments slightly longer
F	No 9 (March 2)	2 06	2 0	1 50 (2 h 45 m)	—	—	—	Oxygen reduced by small fish
G	No 9 (March 3)	1 84	—	1 73 (30 mins)	1 77 (15 mins)	1 84 (15 mins)	—	Small fish put into experiment tube, no siphoning from jar
H	No 9 (March 5)	1 90	2 23 (30 mins)	1 56 (30 mins)	1 28 (15 mins)	1 11 (15 mins)	—	Oxygen reduced by small fish Much mucus at last test
I	No 11 (March 19)	0 44	0 55	0 27 (30 mins)	0 44 (30 mins)	1 11 (1 hour)	1 22 (1 hour)	Water from pool near Dunas Filaments about 1 5 cm long
J	No 11 (March 20)	—	0 88	1 10 (1 hour)	2 12 (1 hour)	1 22 (1 hour)	—	Water from pool near Dunas Much mucus at last test
K	No 13 (March 21)	0 95	0 88	1 22 (30 mins)	1 22 (30 mins)	2 34 (1 hour)	1 67 (1 hour)	Water from Dunas pool At the 3rd test end of siphon tube raised to level of filaments Longest filaments 2 2 cm. long

Table I —(Continued)

	Number and date	Original water	After Lepidos	1st test	2nd test	3rd test	4th test.	Water and remarks
L	No 13 (March 22)	0 78	0 89	1 45 (1 hour)	2 01 (1 hour)	2 12 (1 h 25 m)	—	Water from Dunas pool.
M	No 13 (March 23)	1 56	1 85	2 34 (1 h 5 m)	3 01 (1 hour)	—	—	Water from Dunas pool. At end of 2nd test, end of siphon tube opposite filaments.
N	No 13 (April 2)	—	2 01	2 23 (35 mins)	—	—	—	Experiment made in laboratory of Muséu Commercial, Belem Water, oxygen reduced by small fish
O	No 13 (April 4)	—	0 89	1 11 (25 mins)	1 72 (3½ hours mean of 2 tests)	—	—	Muséu Commercial. Reason for long interval was that laboratory was shut from 11 0 a m to 2 0 p.m

Table II —Experiments on Females Winkler Method Oxygen, cubic centimetres per litre

	Date							
A	February 11	—	2 28	2 15 (30 mins)	1 19 (1hour)	—	—	Well water, oxygen reduced by tamuata.
B	February 24	0 78	1 00	0 83 (25 mins.)	0 11 (30 mins)	—	—	Well water as before At second test, solid mucus in water
C	March 2	1 17	1 28	0 89 (3 h. 10 m)	—	—	—	Well water as before *Lepidosiren* put into glass jar with tamuata after latter was dead
D	March 4	1 48	—	1 81 (30 mins.)	1 45 (15 mins)	0 95 (15 mins)	—	Well water, oxygen reduced by tamuata.
E	March 5	—	0 67	0 61 (30 mins.)	—	—	—	As before
F	March 21	0 95	1 56	1 45 (1 hour)	1 28 (1 hour)	—	—	Water from Dunas pool.

Experiments were therefore made to obtain evidence on this point The results are given in Table III

Table III —Effect of Mucus from *Lepidosiren* on the Oxygen Content of Water

		Cubic centimetres of O_2 per cent			Cubic centimetres of O_2 per cent
A	Male *Lepidosiren* with very short filaments in well water 1 hour, 100 c c drawn off, shaken up with air and tested	3 4	C	Water with female *Lepidosiren* 1¾ hours, 100 c c drawn off, shaken up violently, left to stand ½ hour and tested	2 1
B	Well water direct from well shaken up with air	4 5	D	Well water left standing ½ hour	4 3

Comparison of A and B shows that the presence of mucus reduces the quantity of oxygen indicated even when excess of air has been introduced This must be due either to the absorption of iodine or to rapid absorption of oxygen by the mucus Comparison of A and C shows that the effect is largely due to absorption of dissolved oxygen Comparison of B and D shows that standing half an hour without mucus has very slight effect The reduction in the indicated oxygen after contact with the fish for 1 hour, that is the difference between A and B, is 1 1 c c The sample C was with the fish ¾ hour longer than sample A, therefore the difference between C and A, if due to additional mucus only, should have been 0·8 c c, while it was actually 1 3 c c, a difference of 0 5 c c due to oxidation of the mucus by dissolved oxygen

The contrast between the results of the experiments with male and female is most conspicuous, compare male No 13, March 21 and March 22 (Table I) with female, March 21 (Table III) The water used in these experiments was from the same source and the initial oxygen content almost the same That the oxygen after introduction of the fish rises more in the case of the female is merely due to a difference in the amount of air taken in by the fish After the fish was put in, the oxygen shows a slight but continuous decrease in the female and a striking increase in the male with filaments

Whether, in the Winkler method, the effect of the mucus is to absorb dissolved oxygen or only to absorb iodine, we believe that it is the cause of the decrease in the amounts of oxygen found in the experiments on males with undeveloped filaments, and females The results obtained with males No. 11 and No. 13 which had the pelvic filaments more developed are in striking contrast to those from the other males and from females. In the case of No. 11, March 19, the decrease at the first test was in all probability due to excess of mucus, the abundance of which was noted at the time, and the same is true of the last test on March 20. Otherwise there was a steady increase of the amount of oxygen with time. The figures for No. 13 are still more striking. The experiments were made on three successive days with similar results and a remarkable increase in the oxygen content occurred when the end of the siphon, by which the sample was drawn off from the tube, was raised above the layer of mucus to the level of the pelvic filaments of the fish. That the oxygen content should be increased from less than 1·0 to over 2·0 c.c. per litre and from 1·85 to over 3·0 c.c. is, we think, very remarkable, especially when it is considered that this occurred in the presence of mucus which would seem to cause a decrease in the amount of oxygen. It is obvious that if the filaments were respiratory organs performing a respiratory function, the dissolved oxygen should have been more reduced when they were present than when they were absent. The results of the experiments are shown in the form of curves in the graph (fig. 1). We consider that these results afford positive evidence that the pelvic filaments of the male *Lepidosiren* emit oxygen when the fish is surrounded by water in which the dissolved oxygen is reduced to between 0·5 c.c. and about 2 c.c. per litre.

When Mr. Cunningham left Dunas on March 25 he took with him six *Lepidosiren* alive, three females and three males, one of the latter being No. 13. When he arrived in Belem he deposited them, by the kind permission of the Director of the Muséu Goeldi, in a tank in the Zoological Garden attached to that institution. By permission of the Director he was allowed to work in the laboratories of the Museum Commercial of Belem, and he repeated the experiments on specimen No. 13 with apparatus and reagents brought from Dunas. The special tubes used had been broken in the course of the work and the filaments of the fish had suffered some damage in transit to Belem, but a suitable jar was obtained. The dissolved oxygen content was again reduced by the respiration of a small fish as in the experiments at Dunas. Owing to the circumstances the experiments were not so complete or satisfactory as those previously made, but two were carried out which showed increase of dissolved oxygen as recorded in the table.

We have not attempted to measure the absolute amounts of oxygen given off or absorbed in our experiments, our figures are all relative or comparative, but it may be of some interest to give here the dimensions of the tubes and specimens with which the experiments were made, and a rough estimate of the quantities of water tested The tube most frequently employed was 75 cm long and 6 25 cm in diameter Its capacity therefore was 2415 6 cm, or a little less than 2½ litres, but it was only filled to about half its height to allow for the volume of the fish The initial quantity of water was therefore about 1¼ litres If we take the experiment on No 13, March 22, as an example, the amount of oxygen added to the water in 1 hour is about 0·56 c c per litre.

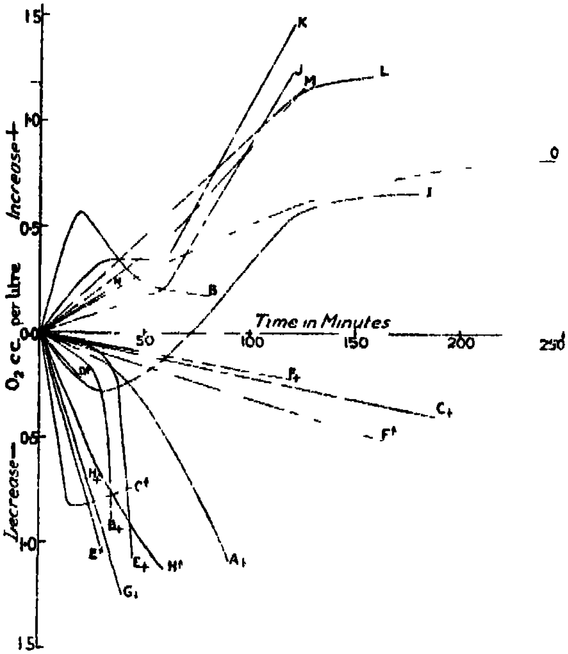

FIG 1—Curves showing the increase or decrease of dissolved oxygen in water containing *Lepidosiren* The zero point is the amount found immediately after the introduction of the fish, the other points are obtained by deducting the initial amount from the amounts found after successive intervals of time

Males with well developed filaments	A, B, etc
Males with incipient filaments	A↑, B↑, etc
Females	A^+, B^+, etc

Allowing for the 50 c c drawn off immediately after the fish was put in, we find that 0·812 c c of oxygen was given off by the fish in the first hour The figure is too large on account of the fact that the concentration was greater in the neighbourhood of the filaments, and too small on account of the effect of the mucus But the absolute amount of oxygen given off by the fish in a given time is of less importance than its ability to raise the concentration of oxygen in the nest to a degree at which respiration of the eggs and young can take place. Carter and Beadle give reasons for concluding that this concentration is lower in the case of *Lepidosiren* and *Symbranchus* than in that of other fishes

Temperature —We made no systematic observations on the temperature of the water in the marshes or igarapés, but we give below the maximum and minimum temperatures observed within the house at Dunas, and as the windows and doors were nearly always open, with the wind blowing through them, these temperatures do not differ much from shade temperatures taken out of doors As our analyses were carried out in a store-room forming part of the ground floor of the house, the figures also indicate approximately the temperatures of the water with which the experiments on *Lepidosiren* were made

Temperatures, in Degrees Centigrade, taken by Registering Thermometer, at Dunas

Date	Minimum.	Maximum	Date	Minimum	Maximum
January 24	25 2	—	February 22	24 7	28 6
January 26	25 2	30 8	February 23	25 2	28 8
January 27	27 2	31 3	February 24	23 3	30 5
January 28	In sun outside	44 4	March 6	21 6	26 9
			March 14	25 8 (6 30 a m)	—
February 8	25 2	28 8			
February 11	24 7	28 6	March 16	24 7	30 5
February 19	24 1	30 5	March 23	23 6 (8 20 a m)	—

Experiments on Symbranchus marmoratus

Agar was in the Gran Chaco in 1907 and collected specimens of embryological material of *Symbranchus* which he gave to Sister Monica Taylor (1913), together with his field notes, which she quotes in her paper on the development of the larva According to these notes the breeding burrow is a tunnel running obliquely downwards into the mud at the bottom of the swamp, and the adult male was always found in it with the eggs. The habits, therefore, are closely similar to those of *Lepidosiren*, and the same two questions arise (1) What is the mode of respiration of the adult ? (2) How are the eggs and larvæ in the nest supplied with oxygen ? With regard to the first question it has been generally concluded, since the fish æstivates in the dry season and no special organ of aerial respiration has been discovered, that in *Symbranchus* the branchial organs perform this function in the absence of water or of sufficient oxygen in the water Monica Taylor refers to the great length of the branchial chamber its small single ventral opening, and the vascular character of the lining as adaptations to aerial respiration, but we have not seen any mention of observations on visits of the fish to the surface to breathe air while living in the water

On January 29 we obtained two specimens of *Symbranchus* at the head of the Rego do Jacaré magro They were dug out of burrows near or in the bank of the igarapé, and were taken separately Both were mottled with black spots, on ground colour which in the larger of the two was yellow, and in the other red They were alive when brought to the house in the evening and were placed in a kerosene tin with only a little water, as it was thought that they would live well by aerial respiration, but the next morning one was dead and the other moribund Efforts were made to restore the latter by artificial respiration When a finger was placed in the branchial aperture the fish reacted by one or two full inspirations, in which the chamber was very much dilated The thin wall of the chamber, destitute of opercular bones, was thrown into folds when collapsed, and distended to a globular form when filled with air After 2 hours, however, when the fish seemed to be recovering, it suddenly ceased to react and died The larger specimen was a female, 121 cm long and 16 5 cm in circumference at its maximum thickness just behind the head The branchial cavity was very long and wide, and when cut open the lining membrane was seen to be of a deep red colour, almost as red as the gills The latter were four in number with a double series of filaments on each arch, as in other fishes There were no special cavities or folds on the mucous membrane of the operculum but that of the palate was thick and folded, with a marked ridge along the bone running parallel to the upper jaw A depression on the roof of the pharynx between the upper ends of the gill-arches seemed to have little importance The whole body of both specimens was extremely vascular, and all the tissues were well supplied with blood, the quantity of which was unusually large *Symbranchus* has no air-bladder, and it may be inferred that its aerial respiration was evolved after its bladder had been lost, whereas *Lepidosiren* has always retained the function of aerial respiration which was the original function of the bladder or lung

The other, slightly smaller, specimen was distinguished by the redness of the ground colour of the skin, which seemed to be due to vascularity, in small scattered areas the colour was deeper The specimen was apparently male, with short, vascular testis The latter had, however, been discharged recently, and when a small piece was teased out and examined under the microscope, no spermatozoa could be recognised

On March 21 another living *Symbranchus* was brought to us together with specimens of *Lepidosiren* from Bonito. An attempt was made to carry out some experiments with this specimen to try to find out how oxygen was conveyed to the eggs and young in the burrow in this species It seemed possible that the fish might enter the burrow with its branchial cavity full of air, or that when it passed water through the cavity some oxygen would be given off by the gills and vascular lining, which had just been exposed to the air in a visit to the surface The specimen was larger than those already mentioned, and, unlike them, was of uniform colour, almost black It was put head downwards into one of the large glass tubes containing water with less than 1 c c per litre of oxygen The branchial cavity was collapsed and only very slight branchial movements were observed at long intervals Only one minute bubble of air was seen to escape The result as seen in Table IV was marked diminution of the amount of dissolved oxygen, as with a female *Lepidosiren* A second experiment showed a similar result, but the oxygen found after 1 hour could only be compared with the original water as no test was made immediately after the fish was put in The original water used in the second experiment contained a much higher content of oxygen than that used in the first, obtained as it was after a night of heavy rain The reduction of oxygen in this case might be attributed to aquatic respiration, except that the tension of the oxygen in the blood of the fish was probably, at any rate at first, higher than in the surrounding water It is probable that the reduction was due to cutaneous mucus as in *Lepidosiren* These experiments give no support to the suggestion that the branchial organs can give off oxygen to deoxygenated water

The *Symbranchus* was then placed in the tube with its head upwards and above the surface of the water, and it expanded its huge branchial cavity with air at intervals It was noticed that the anal aperture at the lower end of the tube was unusually dilated and the inner surface of the red vascular rectum to some extent exposed It seems possible that oxygen is given off to deoxygenated water by this rectal membrane, the blood in the circulation having been fully oxygenated by aerial respiration at the surface No evidence of emission of oxygen in any other way was obtained, neither was the sex of the specimen determined, as no more time or attention could be spared for the study of *Symbranchus* It is to be hoped that experimental investigations with this fish will be carried further by other biologists in the near future

Table IV — Experiments with *Symbranchus marmoratus*. Dissolved Oxygen in cubic centimetres per litre

Date	Original water	Immediately after fish was put in	1st test	Water and remarks
March 22	0 78	1 23	0 44 (1 hour)	Water from Dunas pool. Fish put into tube head downwards. Respiratory movements at ventral branchial opening very slight and at long intervals. Only one minute bubble of air seen to escape
March 23	2 9	—	2 3 (1 hour)	Water from same pool after much heavy rain. Fish head downwards as before

Summary

(1) We arrived in the island of Marajo on December 18, 1930, before the end of the dry season, and it was several weeks before we obtained specimens of *Lepidosiren*. We found that they were widely distributed in the marshes of the north coast of the island, but difficult to capture and not abundant. We found that towards the bottom of the stagnant water in these marshes there was a deficiency of dissolved oxygen, as found by Carter and Beadle in the swamps of Paraguay.

(2) When specimens of *Lepidosiren* were placed in water with a very low content of dissolved oxygen, it was found that the quantity of oxygen decreased after successive intervals of time in the case of females, and usually that of males with only incipient filaments, but a distinct increase occurred in the case of males with well-developed filaments.

(3) The possible action of the mucus secreted by the fish in causing decreases in the content of dissolved oxygen, as determined by the Winkler method, is discussed. As the mucus is equally secreted by males with well-developed filaments, it is concluded that the increase correlated with the presence of the filaments is evidence of the emission by those structures of a greater quantity of oxygen than the figures actually show.

(4) We obtained a few specimens of *Symbranchus* and made two experiments which showed a decrease of dissolved oxygen as in the case of *Lepidosiren*. But we had no time to pursue further the investigation of this species.

Analyses have since been made, at the Laboratory of the Aquarium of the Zoological Society's Gardens, of water which had been in contact with living *Lepidosiren* for varying periods, the specimen used being one of three which were brought home alive by Mr Cunningham and deposited in that Aquarium. The objects of these analyses were to find (1) the reduction in the quantity of dissolved oxygen which occurred when the water was left for a period at different temperatures after separation from the fish, (2) how much of the reduction in the quantity of dissolved oxygen indicated by the Winkler method after contact with *Lepidosiren* was due to actual loss of oxygen, and how much to direct absorption of iodine by the mucus, (3) what was the effect of blowing air through the water in which the amount of dissolved oxygen had been reduced. It was shown that oxygen as well as iodine is absorbed by the mucus, and that aeration raises the quantity of dissolved oxygen to saturation point or above that point.

Acknowledgments

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The Antiseptic and Trypanocidal Action of certain Styryl and Anil Quinoline Carboxylamides *

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In the case of quaternary compounds of the styryl quinoline series and of the analogous benzthiazole derivatives a powerful trypanocidal effect *in vivo* has been shown to depend on the presence in the substance of a free basic group in one of the nuclei, and an acylamino (especially acetyl) or urethane group in the other, and also on the styryl linkage—each playing a definite part in contributing to the action (Browning, Cohen, Ellingworth and Gulbransen, 1929, 1931) This is exemplified by 2(*p*-aminostyryl)-6 acetylamino quinoline methochloride (No 8), 2(*p*-dimethylamino styryl)-6 acetylamino quinoline methochloride (No 25), 2(*p*-acetylamino)-6 dimethylamino quinoline methochloride (No 90) and 2(*p*-dimethylamino styryl) quinolyl (6) urethane (Me) methochloride (No 125) The effect of acetylation of the amino group parallels that discovered by Ehrlich and his co-workers in the case of *p*-amino phenyl arsinic acid Gough and King (1930) have recently made the important observation that in the latter series the introduction of an amide group converts the therapeutically inactive carboxylic and sulphonic acids into active compounds Accordingly, the effect of substituting a carboxylamide group for the acylamino in compounds of the type of No 25 and its anil analogue (No 62) has been investigated. In addition, the position of the carboxylamide group has been varied. These substances were further examined for antiseptic action, the results being shown in the table

Trypanocidal properties have been tested on *T brucei* infections in mice as in previous work. The striking observation has been made that only those compounds with the carboxylamide group in the 6 position are therapeutically active, the anils being only slightly less effective than the styryl analogues (*cf.* Nos. 410, 409 and 385, 403) This contrasts with what is found in the acetylamino derivatives, since the styryl compounds of the latter are highly active as compared with the corresponding anils The carboxy-ethylamides (420, 419) are more toxic and less trypanocidal than the corresponding amides and methylamides

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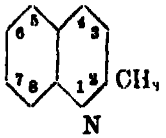
Antiseptic properties—Antiseptic action toward *Staphylococcus aureus* and *B coli* in peptone water and serum media was examined as previously described (Browning, Cohen, Ellingworth and Gulbransen, 1926, 1928) The concentration which produces sterility or, failing this, causes marked inhibition of growth of the organisms, has been indicated in the table, since this value serves as a suitable measure of the antiseptic power of such compounds, as previously pointed out In general, these substances are not powerful antiseptics, although certain of them are fairly active, *e g*, Nos 403, 419, 413, 420. But nothing approaching the powerfully active acylamino derivatives of the anil quinolines has been met with

Experimental

The formation of the amides described below were obtained by the following series of changes

$$\mathrm{R\ CO\ OH} \xrightarrow{\mathrm{C_2H_5OH}} \mathrm{R\ CO\ OC_2H_5} \xrightarrow{\mathrm{NH_3}} \mathrm{R\ CO\ NH_2}$$

The numbers represent the position of the carboxyl group in the quinaldine nucleus



Quinaldine 6 carboxylic acid was prepared by the method of Doebner and Miller (1884) by heating on the steam-bath for 2 hours a mixture of *p*-aminobenzoic acid, conc hydrochloric acid and paraldehyde The yield is about 50 per cent of the original material It darkens at 240° and melts at 259° (decomp)

Quinaldine 6 carboxylic ester is readily obtained by suspending 12 grams of the finely powdered acid in 150 c.c of saturated alcoholic hydrogen chloride for 24 hours at 40°–50° It forms a pale brown solution, which, on distilling off the alcohol under diminished pressure, leaves a viscid liquid, consisting of the hydrochloride, which quickly solidifies It is dissolved in water, precipitated with sodium carbonate and the solid ester filtered and washed with cold water. The dried product may be crystallised from petroleum ether. The yield is 10–11 grams, melting point 57° It forms large colourless prisms

The esters of the other isomeric acids were prepared by the same general method

Quinaldine 6 carboxylamide—Quantities of 4 grams of the ester and 50 c.c. conc ammonia were heated in sealed tubes in the steam-bath for 2–3 days, when the ester was converted into thick, colourless prisms which line the tube The crystals were filtered, washed and recrystallised from a large volume of boiling water The amide separates in the form of colourless needles, melting point 225°–227° The yield from 4 grams was 2 grams of pure amide

The other amides described below were prepared in a similar manner

Quinaldine 6 carboxylamide methosulphate—The following example represents the general method employed for preparing the methosulphates of the amides. The finely powdered amide is placed in a flask and eight times its weight of nitrobenzene is carefully poured in without shaking The mixture is gently heated until the amide just dissolves The flame is removed, the calculated quantity of methyl sulphate added, shaken up and left to cool The methosulphate crystallises It is dissolved in water, freed from nitrobenzene by frequent extraction with ether and the solution evaporated It can be recrystallised from alcohol and forms colourless needles

Quinaldine 6 carboxylamide methiodide.—To obtain the methiodide the methosulphate is dissolved in water, potassium iodide (equal in weight to the amide taken) added and the solution, if necessary, concentrated The methiodide, which is not very soluble, separates in dark orange needles, melting point 230° (decomp) The yield is nearly quantitative The other methiodides were prepared in a similar manner

Quinaldine 6 carboxylamide methochloride is prepared from the methiodide by dissolving the latter in aqueous methyl alcohol and heating on the steam-bath with an excess of silver chloride, prepared by precipitating silver nitrate with hydrochloric acid and washing with hot water and then alcohol until free from acid The methiodide and silver chloride in aqueous methyl alcohol were boiled on the steam-bath for 3 hours, filtered and evaporated. The residue consisted of colourless crystals of the methochloride. This method is a general one for converting methiodides into methochlorides

Quinaldine 6 carboxymethylamide—The ester was heated with methylamine (33 per cent) in a sealed tube for 2 days in the steam-bath. It forms colourless, striated crystals, melting point 202°–204°.

Quinaldine 6 carboxymethylamide methosulphate forms thin, colourless prisms. The methiodide crystallises from water in pale yellow needles or prisms with square ends.

Quinaldine 6 carboxyethylamide prepared from the ester and ethylamine (33 per cent.) crystallises from a concentrated aqueous solution in long, colourless prisms, melting point 153°. The methiodide crystallised from alcohol in clusters of yellow needles, melting point 241°–242° (decomp.).

Quinaldine 5 carboxylic acid was prepared by the method of Doebner and Miller (1884). *The ester* crystallised from alcohol in long needles, melting point 72°. Found N, 6·8 per cent., $C_{13}H_{13}O_2N$ requires N, 6·5 per cent.

Quinaldine 5 carboxylamide.—A mixture of the ester (4 grams) conc. ammonia (40 c.c.) and alcohol (10–15 c.c.) was heated in a sealed tube in the steam-bath for 3 days. The amide crystallises from boiling water in long prisms, melting point 249°. Found N, 14·9 per cent., $C_{11}H_{10}ON_2$ requires N, 15·05 per cent. Owing to the solubility of the methiodide obtained by the nitrobenzene method, the aqueous solution was evaporated nearly to dryness on the steam-bath and then dried in a desiccator. The solid was powdered and extracted with a little cold acetone, which removed some dark-coloured impurity and the yellow residue was boiled with methyl alcohol and filtered. The crystalline solid obtained on cooling the filtrate was recrystallised from a small quantity of water containing some potassium iodide and finally crystallised from methyl alcohol. The methiodide forms yellow plates, melting point 233°–234° (decomp.). Found I, 37·9 per cent., $C_{12}H_{13}ON_2I$ requires I, 38·7 per cent.

Quinaldine 5 carboxymethylamide forms clusters of long needles, melting point 176°. Found N, 14·3 per cent., $C_{12}H_{12}ON_2$ requires N, 14·0 per cent. The methiodide was prepared by the nitrobenzene method and also by the method described under the 3 isomeride. It dissolves in water and crystallises on concentration of the solution in a vacuum desiccator. It crystallises from alcohol in yellow prisms, melting point 238°–239° (decomp.). Found I, 36·7 per cent., $C_{13}H_{15}ON_2I$ requires I, 37·1 per cent.

Quinaldine 4 carboxylic acid was prepared by Pfitzinger's (1897) method. The yield was equal to the weight of isatin taken. In evaporating the mother liquor it is essential to keep the solution alkaline to avoid decomposition. It melts at 250°. The ester crystallises in colourless prisms from petroleum ether, melting point 77°.

Quinaldine 4 carboxylamide crystallises in colourless crystals, melting point 244°. The methiodide crystallises in orange needles, melting point 226°–227° (decomp.)

Quinaldine 4 carboxymethylamide crystallises from dilute alcohol in colourless prisms, melting point 152°–153° Found N, 14·1 per cent, $C_{12}H_{12}N_2O$ requires N, 14 0 per cent The methiodide which was prepared from the methosulphate crystallises in long, orange prisms It melts with decomposition at 227°–228°

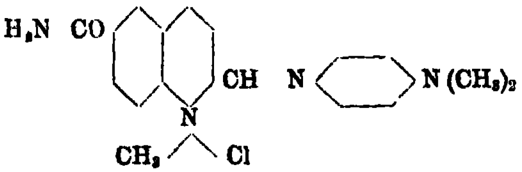
Quinaldine 4 carboxyethylamide crystallises from water in long prisms, which softened at 100° and melted at 125° The methiodide crystallised from water in yellow needles, melting point 112°–113°

Quinaldine 3 carboxylic ester was prepared as described by Friedländer and Gohring (1883) from *o*-aminobenzaldehyde and ethylacetoacetate The methiodide was crystallised from water and melted with decomposition at 210°–211°. Found. I, 35 3 per cent, $C_{14}H_{16}O_2NI$ requires I, 35·6 per cent

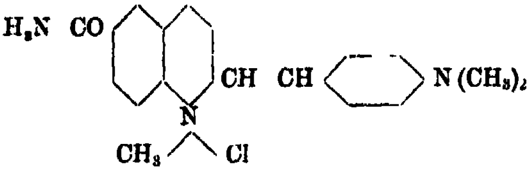
Quinaldine 3 carboxylamide cannot be prepared by the usual method of heating the ester with conc ammonia or with alcoholic ammonia As the corresponding ester is prepared by the condensation of ethyl acetoacetate with *o*-aminobenzaldehyde, it was thought that the amide might be formed by substituting acetoacetamide for the ester, which proved to be the case The acetoacetamide was prepared by the method of Claisen and Meyer (1902) A mixture of *o*-aminobenzaldehyde (5 4 grams), acetoacetamide (4 5 grams), alcohol (6 c c), water (5 c c.) and 7 drops of 10 per cent sodium hydroxide was allowed to stand overnight. The cream-coloured solid was filtered and recrystallised from water The yield was 6 grams It crystallised from water in long needles, melting point 199°–201°. Found N, 15 4 per cent., $C_{11}H_{10}ON_2$ requires N, 15·05 per cent The methiodide crystallised from water in yellow prisms, which gradually darkened on heating and decomposed at 246°–247° Found I, 38 3 per cent, $C_{12}H_{13}ON_2I$ requires I, 38 7 per cent. The methiodide like that obtained from the ester was unstable towards ammonia and did not condense with nitrosodimethylaniline or dimethylaminobenzaldehyde

Quinaldine 8 carboxylic acid was prepared by the method of Doebner and Miller (1884). One hundred grams of anthranilic acid gave 14–15 grams of the hydrochloride The ester is a liquid and was used without further purification for the preparation of the amide

Quinaldine 8 carboxylamide after recrystallisation from water formed colourless hair-like needles, which melted at 170°–171°. Found. N, 15 4 per cent., $C_{11}H_{10}ON_2$ requires N, 15 05 per cent The methiodide crystallised from water and was recrystallised from dilute alcohol, it darkened on heating at about 230° and decomposed between 245° and 255° Found. I, 39·2 per cent., $C_{12}H_{13}ON_2I$ requires I, 38·7 per cent. The methiodide decomposed with ammonia and, though it did not condense under normal conditions with nitrosodimethylaniline or dimethylaminobenzaldehyde, it did occur with the latter under special conditions. The anil and styryl derivatives obtained from the foregoing by condensation may be represented by the case of the 6-carboxylamide as typical of the series.



2(*p*-dimethylamino anil) quinoline 6 carboxylamide methochloride



2(*p*-dimethylamino styryl) quinoline 6 carboxylamide methochloride

The method of preparation described below in the case of the above two compounds is generally applicable to the other members of the series. The numbers in brackets have reference to the table at the end of the paper.

2(*p-dimethylamino anil*) *quinoline* 6 *carboxylamide methiodide*.—The quinaldine 6 carboxylamide methiodide (2 grams) was dissolved in alcohol and a little water and *p*-nitrosodimethylaniline (1 gram) in alcohol added together with 2 drops of piperidine. It was heated on the steam-bath for ½ hour, cooled, filtered and washed with alcohol. It forms small blue needles with green reflex and dissolves slightly in water and alcohol with a deep blue colour. It can be crystallised from dilute alcohol and separates in clusters of needles. The yield was 2·2 grams.

The corresponding methochloride (385) was prepared in the usual way by boiling the methiodide with silver chloride in methyl alcohol for several hours. The solution was filtered hot and the filtrate cooled. It deposited green iridescent needles which dissolved readily in alcohol and water with a deep purple colour and crystallises from water in needles. Found: Cl, 9·1 per cent., $C_{20}H_{21}N_4OCl$ requires Cl, 9·6 per cent.

2(*p-dimethylamino styryl*) *quinoline* 6 *carboxylamide methiodide* was prepared by heating on the steam-bath quinaldine 6 carboxylamide methiodide (2 grams) with *p*-dimethylaminobenzaldehyde (1 gram) with the addition of 2 drops of piperidine, in aqueous alcoholic solution The liquid, after boiling for several hours and cooling, deposited 0·8 gram of crystals having a bronze reflex They were filtered and washed with alcohol The substance being very slightly soluble in water and alcohol even on boiling was converted into the methochloride, the conversion being carried out in aqueous solution. The aqueous solution on evaporation left a green iridescent residue which dissolved readily in water with a bright magenta colour and from which it separated in an indefinite microcrystalline form Found Cl, 8·9 per cent , $C_{21}H_{22}ON_3Cl$ requires Cl, 9 6 per cent

A better method is to use the methosulphate for condensation The product (401) crystallised in black needles with a bronzy lustre It can be recrystallised from dilute alcohol in the form of dark-coloured needles, which dissolve in alcohol and water with a bright magenta colour like other quaternary salts of the styryl compounds

2(p-dimethylamino anil) quinoline 6 carboxymethylamide methiodide was prepared from the 6 carboxymethylamide and nitrosodimethylaniline The anil separated in needles with metallic reflex It dissolves in alcohol and to some extent in water with a bluish purple colour Found I, 27 0 per cent. , $C_{21}H_{23}ON_4I$ requires I, 26 8 per cent The methiodide was converted into the methochloride (403), which separates as a crystalline magma and dissolves in alcohol and water with a deep blue colour.

2(p-dimethylamino styryl) quinoline 6 carboxymethylamide methosulphate (409) crystallises from hot water in clusters of needles with bronze reflex and dissolves in both alcohol and water with a bright magenta colour Found S, 6·85 per cent , $C_{23}H_{27}O_5N_3S$ requires S, 7 01 per cent

2(p-dimethylamino anil) quinoline 6 carboxyethylamide methiodide crystallises from alcohol in dark purple needles with green reflex The alcoholic solution is blue. Found I, 25 7 per cent , $C_{22}H_{25}ON_4I$ requires I, 26 0 per cent The methochloride (419) is soluble in alcohol and water with a blue colour

2(p-dimethylamino styryl) quinoline 6 carboxyethylamide methiodide crystallises from alcohol in dark needles with green iridescence and dissolves in alcohol with a magenta colour Found I, 25·8 per cent , $C_{23}H_{26}ON_3I$ requires I, 26·1 per cent The methochloride (420) is soluble in water with a magenta colour.

2(p-dimethylamino anil) quinoline 5 carboxylamide methiodide (400).—The substance was recrystallised from 50 per cent alcohol containing a small quantity of potassium iodide and then from 50 per cent alcohol It forms deep blue prisms with a green metallic lustre and dissolves in water with a purple colour Found · I, 27·1 per cent , $C_{20}H_{21}ON_4I$ requires I, 27·6 per cent

2(*p-dimethylamino styryl*) *quinoline* 5 *carboxylamide methiodide* (401) was recrystallised from 50 per cent alcohol containing a little potassium iodide and then from 50 per cent alcohol It forms long needles with green lustre and dissolves in water with a magenta colour Found I, 27·4 per cent , $C_{21}H_{22}ON_3I$ requires I, 27 7 per cent

2(*p-dimethylamino anil*) *quinoline* 5 *carboxymethylamide methiodide* crystallises from alcohol in clusters of purple needles with green reflex Found I, 26·4 per cent , $C_{21}H_{23}ON_4I$ requires I, 26·8 per cent The methochloride (406) dissolves in water with a bluish purple colour and crystallises on slow evaporation like the methiodide

2(*p-dimethylamino styryl*) *quinoline* 5 *carboxymethylamide methiodide* crystallises from alcohol in purple brown needles with green lustre Found I, 26 5 per cent , $C_{22}H_{24}ON_3I$ requires I, 26·85 per cent The methochloride (413) gave a magenta solution in water and deposited needles from methyl alcohol solution

2(*p-dimethylamino anil*) *quinoline* 4 *carboxylamide methiodide* —The methiodide forms needles with green reflex, which dissolve, though not readily, in water and alcohol with a deep purple colour Found I, 27·1 per cent , $C_{20}H_{21}ON_4I$ requires I, 27 6 per cent The methochloride (395), which is more soluble, crystallises like the methiodide and dissolves with the same purple colour

2(*p-dimethylamino styryl*) *quinoline* 4 *carboxylamide methiodide* (393) forms steely blue needles, which were crystallised from methyl alcohol It dissolves in water with a magenta colour Found I, 27 4 per cent , $C_{21}H_{22}ON_3I$ requires I, 27 7 per cent

2(*p-dimethylamino anil*) *quinoline* 4 *carboxymethylamide methiodide* crystallises in needles which dissolve in alcohol and water with a deep purple colour It crystallises from water in dark blue prisms Found I, 26 3 per cent., $C_{21}H_{23}ON_4I$ requires I, 26 8 per cent It was converted into the methochloride (405) which forms green iridescent needles soluble in water with a deep blue colour

2(*p-dimethylamino styryl*) *quinoline* 4 *carboxymethylamide methiodide* was obtained in the form of steel blue needles or long prisms with square ends, which dissolve in alcohol with a magenta colour, slightly soluble in water Found: I, 26 1 per cent , $C_{22}H_{24}ON_3I$ requires I, 26·8 per cent The methochloride crystallises from methyl alcohol like the methiodide and dissolves in alcohol and water

2(p-dimethylamino anil) quinoline 4 carboxyethylamide methiodide (414) crystallises from dilute alcohol in dark blue needles The aqueous and alcoholic solutions are blue Found I, 26·2 per cent., $C_{22}H_{25}ON_4I$ requires I, 26·0 per cent

2(p-dimethylamino styryl) quinoline 4 carboxyethylamide methiodide (418) crystallises from dilute alcohol in clusters of purple brown needles with green reflex The aqueous and alcoholic solutions were bluer in colour than the other members of the series Found I, 25 65 per cent , $C_{23}H_{26}ON_3I$ requires I, 26·1 per cent

2(p-dimethylamino anil) quinoline 3 carboxylic ethyl ester methiodide —Crystallised from alcohol, it forms clusters of purple needles Found I, 25 6 per cent , $C_{22}H_{23}O_2N_3I$ requires I, 26 0 per cent The methochloride crystallises in needles like the methiodide The colour of the solution was a reddish blue

2(p-dimethylamino styryl) quinoline 3 carboxylic ester methiodide —When crystallised from aqueous alcohol it forms purple needles and in warm water gives a reddish magenta solution Found I, 25 6 per cent , $C_{23}H_{25}O_2N_2I$ requires I, 26 0 per cent

2(p-dimethylamino styryl) quinoline 8 carboxylamide methochloride —An intimate mixture of the amide methochloride (1 gram) and *p*-dimethylaminobenzaldehyde (0·6 gram) was heated to 140°–150° for about 5 minutes It melted, frothed and finally solidified. The solid was extracted with about 50 c c of boiling 50 per cent alcohol, and after filtration the methiodide crystallised in purple needles. The yield was 0·65 gram. It was converted into the methochloride, which had the same appearance as the methiodide Found . Cl, 9 2 per cent , $C_{21}H_{22}ON_3Cl$ requires Cl, 9·6 per cent It dissolves in alcohol and aqueous alcohol with a purple colour , but on dilution the colour disappears

No.	Substance	Antiseptic action.				Precipitation.		Trypanocidal action	
		Staphylococcus aureus		*B coli*					
		Peptone water	Serum.	Peptone water	Serum	In peptone water.	In serum.	Dose	Result
385	2(*p*-dimethylamino anil) quinoline 6 carboxylamide MeCl	20	4	20	10	10	2	1/300–1/400 1/500–1/600 1/750–1/2500	Cure (Cure) Marked
403	2(*p*-dimethylamino anil) quinoline 6 carboxymethylamide MeCl	40	100	10	10	4	2	1/500–1/1000 1/2500 1/5000	Cure (Cure) Slight trace
419	2(*p*-dimethylamino anil) quinoline 6 carboxyethylamide MeCl	40	20	<1	20	10	2	1/2000 1/5000	Slight 0
400	2(*p*-dimethylamino anil) quinoline 5 carboxylamide MeI	10	4	2	4	—	—	1/750	0
406	2(*p*-dimethylamino anil) quinoline 5 carboxymethylamide MeCl	40	10	2	4	—	—	1/1000	0
396	2(*p*-dimethylamino anil) quinoline 4 carboxylamide MeCl	2	4	<1	4	—	—	1/600	0
405	2(*p*-dimethylamino anil) quinoline 4 carboxymethylamide MeCl	4	2	<1	2	2	—	1/500	0
414	2(*p*-dimethylamino anil) quinoline 4 carboxyethylamide MeI	2	2	<1	<1	—	—	1/1500	0
404	2(*p*-dimethylamino styryl) quinoline 8 carboxylamide MeCl	2	<1	2	<1	20	4	1/100	0

410	2(p-dimethylamino styryl) quinoline 6 carboxylamide Me_2SO_4	<1	20	<1	<1	20	2	1/250–1/2500 1/5000 1/10000	Cure Marked Trace
409	2(p-dimethylamino styryl) quinoline 6 carboxymethylamide Me_2SO_4	20	20	<1	4	4	2	1/500–1/5000 1/10000	(Cure Marked
420	2(p-dimethylamino styryl) quinoline 6 carboxyethylamide MeCl	100	40	<1	40	10	—	1/5000	Slight
401	2(p-dimethylamino styryl) quinoline 5 carboxylamide MeI	10	4	<1	4	—	—	1/500	Slight
413	2(p-dimethylamino styryl) quinoline 5 carboxymethylamide MeCl	400	40	20	40	20	—	1/3000	0
393	2(p-dimethylamino styryl) quinoline 4 carboxylamide MeI	<3	3	<3	<3	—	—	1/800	0
406	2(p-dimethylamino styryl) quinoline 4 carboxymethylamide MeCl	1	4	<1	<1	10	2	1/500	0
418	2(p dimethylamino styryl) quinoline 4 carboxyethylamide MeI	40	1	<1	<1	—	—	1/600	0

Antiseptic action —The numbers are the reciprocals ÷ 1000, of the concentrations which produce after 48 hours at 37° C sterility or, failing this, inhibition of growth to such a degree that the medium is only faintly turbid or when unclouded yields scanty growth in sub-culture.

Precipitation.—The numbers are the reciprocals ÷ 1000, of the lowest concentrations which cause precipitation in the media.

Trypanocidal action.—The doses shown are reckoned per 20 grams body-weight of the mouse The highest dose shown for each substance is one which approximates to the largest amount borne by uninfected animals without producing obvious toxic effects, e g , loss of weight

The terms used to designate the lesser degrees of trypanocidal action are as follows —

Trace = prolongation of life for several days beyond that of the untreated controls
Slight = disappearance of parasites from the blood for several days to a week
Marked = absence of parasites from the blood for 10 days or longer
(Cure) indicates that with the doses shown cure was effected only in a proportion of the animals treated

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A Comparative Study of the Phosphagens, with some Remarks on the Origin of Vertebrates.

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Introduction

The original discovery of labile phosphorus in muscular tissue by Eggleton and Eggleton (1927, *a*, *b*) was rapidly followed by a succession of researches which demonstrated its great importance in muscular contraction Fiske and Subbarow (1929) showed shortly afterwards that the phosphagen of the Eggletons was a compound containing equimolecular amounts of creatine and phosphoric acid, which, owing to its great instability in acid solution, had always previously been estimated as inorganic orthophosphate It remained for the Eggletons (1928), Nachmansohn (1928, 1929, *a*, *b*) and Meyerhof (1930) to delineate the functional importance of phosphagen in the muscle machinery, its breakdown under anaerobic conditions, its restitution in oxygen, and its energetical relations with the lactic acid cycle.

In the course of their investigations, Eggleton and Eggleton (1928) examined the tissues of a certain number of invertebrates, and failed to find there any creatine phosphate About the same time, however, Meyerhof and Lohmann

(1928) reported the discovery of another phosphagen in crustacean muscle, a phosphagen in which the creatine portion of the molecule was replaced by arginine This led to an examination of certain other invertebrates by Meyerhof himself (1928), from which a wide distribution of arginine phosphate in the invertebrate Phyla was suggested. Throughout the subsequent discussions it has been assumed that creatine phosphate may be regarded as characteristic of the Vertebrates and arginine phosphate as characteristic of the Invertebrates, although in the absence of thorough-going comparative researches, this view could only remain a very tentative generalisation "The development of creatine phosphoric acid in the Vertebrates from the arginine phosphoric acid of the Invertebrates," said Meyerhof (1930, p 93) "must be regarded as a characteristic chemical mutation" It is probable that Meyerhof was led to this sharp division by the general conclusions at which Hunter arrives in his monograph on creatine and creatinine (1928) Reviewing the various attempts which have been made to demonstrate the presence of creatine or creatinine in invertebrates (*e g*, Wilson (1914), Myers (1920)) he points out that nearly all depend on the very unspecific Jaffe reaction, and concludes that up to the present time no good evidence exists in favour of their presence But this is clearly only a negative conclusion, and the issue is therefore prejudged when Meyerhof writes, "Corresponding to the restriction of creatine to the vertebrate group, Eggleton and Eggleton failed to find phosphagen in the muscles of invertebrates" (1930, p 92) Moreover, it is not possible to include all the data in the literature in Hunter's condemnation, for Quast (1924) in reporting the presence of creatinine in the kidney of the gastropod, *Cyclostoma elegans*, states that he relied upon the tests of Weyl and Maschke as well as on that of Jaffe And in any case, there remain large and important groups of animals (the Echinoids for instance) which have never been thoroughly examined either for creatine or for phosphagen

The studies which we describe in the present paper, then, were initiated, firstly, to determine whether the strict correlation of creatine phosphate with Vertebrates and arginine phosphate with Invertebrates could really be maintained in the light of fuller information than that hitherto available. But, secondly, it was to us a matter of interest to observe how far down in the scale of organised creatures the existence of phosphagen could be demonstrated, and with this end in view we examined several accelomate types. Thirdly, if a sharply defined "chemical mutation" does indeed occur at the vertebrate level, it becomes of interest to identify the position of such forms as are believed to represent the most primitive Vertebrates, or forms transitional between

Vertebrates and Invertebrates Of these, the cephalochordate *Amphioxus lanceolatus* is one of the best known, and this, being investigated both by the Eggletons (1928) and by Meyerhof (1928), was found to contain creatine phosphate and not arginine phosphate But the much more abundant group of tunicates or sea-squirts, the Urochorda, which in their larval form (the "ascidian tadpole") possess a well-developed notochord, had not, before our researches, been examined, nor was anything known of the form of phosphagen possessed by the still more interesting Hemichorda, *e g*, *Balanoglossus* This latter group in particular merited study, in view of the central position which it occupies in the theory of the relation between Echinoderms and Vertebrates, and the question of the origin of the latter Phylum

It may be convenient at this point to refer to Table I, which gives a summary of the position before our researches were begun It will be seen that the Eggletons, choosing one or two representatives each from the Cœlenterates, Oligochætes, Crustaceans, gastropod and lamellibranch Molluscs, and the Holothurians, found creatine phosphate to be absent from all *Amphioxus*, however, and all the Vertebrates studied, gave positive results Then following Meyerhof and Lohmann's discovery of arginine phosphate in crustacean muscle, Meyerhof obtained evidence of it in podaxonian body-wall muscle (curiously unrepresentative of the Annelids), in the striped adductor muscle of *Pecten*, and in the longitudinal muscles of Holothurians On the other hand, it was absent from smooth molluscan adductor muscle, from the muscle of the cephalopod mantle, and from the ring-muscles of Holothurians At this point our survey began

Technique

The method of estimation of phosphagens used in the greater number of our experiments was that of direct separation under ice-cold conditions First introduced by Fiske and Subbarow (1929), this method has been successfully applied to many different tissues by Irving and Wells (1928), Gerard and Wallen (1929), and Needham and Needham (1930)

The material was always well cooled in ice before being dissected and weighed. The weighed portion was then thoroughly ground up with ice-cold 5 per cent. or 10 per cent trichloracetic acid, and ice-cold quartz sand in the proportion of 10 c c to 1 gm. of the material After waiting 10 minutes to allow of complete extraction, the mixture was filtered through a cooled funnel and the filtrate neutralised to phenol-phthalein with ice-cold phosphorus-free saturated caustic soda. 4 c.c of the neutralised filtrate were then pipetted out into a small cooled centrifuge tube and treated with 1 c.c. of a 10 per cent. calcium

Table 1—Distribution of the Phosphagens in Invertebrates, according to previous Workers

	Eggleton and Eggleton (1928) creatine phosphate		Meyerhof and Lohmann (1928) arginine phosphate	Meyerhof (1928)		
				Arginine phosphate		Creatine phosphate.
	+	−	+	+	−	+
Protozoa	—	—	—	—	—	—
Coelenterata	—	*Aurelia*	—	—	—	—
Porifera	—	—	—	—	—	—
Platyhelminthes	—	—	—	—	—	—
Nemertinea	—	—	—	—	—	—
Rotifera	—	—	—	—	—	—
Nematoda	—	—	—	—	—	—
Annelida—						
Oligochaeta	—	*Lumbricus*	—	—	—	—
Polychaeta	—	—	—	—	—	—
Hirudinea	—	—	—	—	—	—
Podaxonia	—	—	—	*Sipunculus*	—	—
Arthropoda—						
Antennata	—	—	—	—	—	—
Crustacea	—	Lobster, crab	*Astacus*	—	—	—
Arachnida	—	—	—	—	—	—
Mollusca—						
Gastropoda	—	*Aplysia*	—	—	—	—
Lamellibranchiata	—	*Pecten*, *Mytilus*	—	*Pecten*	*Pinna*, *Cytherea*, *Anodonta*	—
Cephalopoda	—	—	—	—	*Sepia*, *Octopus*	—
Echinodermata—						
Crinoidea	—	—	—	—	—	—
Asteroidea	—	—	—	—	—	—
Echinoidea	—	—	—	—	—	—
Holothuroidea	—	*Holothuria*	—	*Holothuria*, *Stichopus*	—	—
Protochordata—						
Hemichorda	—	—	—	—	—	—
Cephalochorda	*Amphioxus*	—	—	—	*Amphioxus*	*Amphioxus*
Urochorda	—	—	—	—	—	—
Vertebrata—						
Selachia	Dogfish, ray	—	—	—	—	—
Teleostea	Plaice, *Cottus*	—	—	—	—	—
Amphibia	Frog	—	—	—	—	—
Reptilia	Snake, tortoise	—	—	—	—	—
Mammalia	Rabbit, guinea-pig	—	—	—	—	—

chloride solution saturated with calcium hydroxide. After allowing the tube to stand for 10 minutes in ice, it was centrifuged, the centrifugate poured into a 50-c.c graduated flask and the precipitate washed once in the centrifuge with 4 c.c of ice-cold distilled water and 1 c c of the calcium chloride solution. The precipitate, being dissolved in a drop or two of normal sulphuric acid, gave the true preformed inorganic phosphorus, and the filtrate, after having stood at room temperature for 15 minutes in the presence of the molybdate reagent, gave the creatine phosphate phosphorus Next, another portion of 4 c c of the original trichloracetic acid filtrate (before neutralisation) was taken, and sufficient distilled water added to make the concentration of acid about 0 1/N (usually five volumes), since this was shown by Meyerhof (1928) to be the optimum for hydrolysis of arginine phosphate (see fig 6) This was then allowed to remain overnight (15 hours) in an electrically regulated incubator at 28° C after which the estimation of its contents gave us the inorganic plus creatine plus arginine phosphate, and hence by subtraction the latter alone In the tables of this paper the inorganic phosphorus estimation will be referred to as Fraction A, the creatine as Fraction B, the total as Fraction C, and the arginine, C — (A + B), as D Our estimation method throughout was the colorimetric technique of Fiske and Subbarow (1925), a variant of the original Briggs method The molybdate solution used was one of 2 4 per cent ammonium molybdate in 5N sulphuric acid, and the ammonaphthol-sulphonic acid was dissolved in sodium sulphite and bisulphite solution, according to the method of Luck (1929) The standard varied from 0 3 to 0·05 mgm phosphorus in 50 c c and the comparisons were made with a Kober colorimeter Below this level it was necessary from time to time to have recourse to an arrangement of test-tubes of uniform bore, similar to those used in oceanographical investigations, *e g*, by Dienert and Wandenbulcke (1923). We were thus able to deal with amounts as low as 0·01 mgm in 50 c.c , but we do not wish to claim for these figures anything approaching the degree of accuracy which is easily attained with the colorimeter At such dilutions, the judgment of tint was particularly liable to error owing to the presence of substances which gave a slight greenish appearance to the colour Nevertheless some of the figures obtained in the dilute solutions are of considerable interest, and we were often able to observe a definite increase after 15 hours' incubation.

In certain cases we felt it desirable to have an alternative method to the direct separation procedure described above, and we therefore adopted the original extrapolation method of the Eggletons (1927, *a*) The reagents were mixed with the solutions and readings begun as soon as possible afterwards on the colorimeter—in this way sets of data were obtained which could be extrapolated backwards in time to the moment of mixing. Thus we were able to assure ourselves that we were really dealing with a labile compound and not with some failure of the separation method.

For the direct estimation of the creatine we used the method of Walpole (1911) which is based on the diacetyl reaction of Vosges and Proskauer (1898). A portion of the original trichloracetic acid filtrate was neutralised with normal soda, and to 4 c.c. of the neutral extract, 4 c.c. of saturated sodium carbonate solution and 2 drops of a 1 per cent. solution of freshly prepared diacetyl were added. The mixture was then incubated for 1 hour at 37° C. together with a set of standards made up from pure creatine solutions. This method was very satisfactory for creatine, but not for arginine, although we had expected it to estimate both, owing to the guanidine grouping in the amino-acid. The creatine standards formed a good gradation from light to deep pink, but the arginine standards showed little gradation, and this, together with the cloudiness met with among the unknown solutions, made the estimation of arginine impossible.

In order to establish still further the presence of creatine, we converted it to creatinine and estimated the latter. Conversion was effected by heating solutions in an autoclave at 130° for from 20 to 50 minutes, any glucose present having been previously removed by van Slyke's method (1917). To 4 c.c. of the trichloracetic acid extract, 0.25 c.c. of 15 per cent. copper sulphate solution was added, and the mixture neutralised to litmus with strong soda. 0·6 c.c. of a 25 per cent. suspension of calcium hydroxide was then added, and the mixture centrifuged, after which the precipitate was washed once in the centrifuge. The filtrate possessed no blue colour. Estimation of the creatinine was effected by the Jaffe reagents prepared as described by Luck (1929).

Experimental Results.

(1) *Cœlenterata.*—Our investigations of the sea-anemones were not carried as far as we had originally intended; firstly, because the amount of phosphorus present was so low that satisfactory colorimetric estimations could not be obtained, and secondly, because the characteristically slimy nature of cœlenterate tissues made filtration and other operations on the extracts very difficult. Such results as we were able to obtain, however, are shown in Table II, from which it can be seen that arginine phosphate is probably absent from sea-anemones. It is interesting in this connection that Kutscher and Ackermann (1931) could isolate no free arginine, but only guanidine and agmatine from a

Table II—Direct Separation Method

Group.	Animal	Experiment No	Weight of material taken, gm	Mgm P A	Mgm P B	Mgm P C	Mgm P/gm A	Mgm P/gm B	Mgm P/gm C	Mgm P/gm D	Phosphagen in per cent of the total P
Cœlenterata, Actinozoa	*Anthea rustica*—										
	Tentacles	P 1	5·07	0·04	0·00	0·053	0·008	0·00	0·01	0·002	?
	Body-wall	P 2	1·05	0·032	0·00	0·032	0·031	0·00	0·03	0·00	None
	Anthea cereus—										
	Tentacles	AR 1	2·71	0·135	0·00	0·135	0·056	0·00	0·056	0·00	None
Ctenophora	*Pleurobrachia pileus*—										
	Whole bodies	M 1	33·67	0·069	0·00	0·120	0·065	0·00	0·0113	0·0048	42·0
Platyhelminthes, Triclada	*Planaria vitta*	AD 2	0·40	0·042	0·00	0·056	0·106	0·00	0·141	0·035	24·8
	Polycelis nigra	AD 1	0·54	0·084	0·00	0·098	0·155	0·00	0·182	0·027	14·8
Nemertinea	*Lineus longissimus*	V 1	2·10	0·470	0·00	0·978	0·223	0·00	0·467	0·244	52·5
	,,	V 2	1·45	0·245	0·00	0·299	0·169	0·00	0·206	0·037	18·0
	(Whole body in both cases)										

cœlenterate (*Geodia gigas*) Our values for inorganic phosphate in extracts of *Anthea* are of the same order as those of the Eggletons (1928) for *Aurelia*

We were at first inclined to think that the absence of phosphagen could be associated with the fact that Actinozoa, possessing no mesoderm possess no true muscle, and that the myo-epithelial cells (as in the mesenteries of polyps) must have a contraction mechanism which could dispense with phosphagen But this view was contradicted by the results which we obtained on Ctenophores (Table II) Here a considerable proportion of arginine phosphate was present, although apart from the minute contractile thread-like processes which traverse the jelly of the ctenophore, even pre-muscle is not present to any great extent Since the activity of the ctenophore is characterised much more by ciliary motion the question is raised whether arginine phosphate may not be associated with this form of contraction, and it would be interesting to compare the phosphagen content of some ciliated structure during the various phases of activity and under external conditions such as lack of oxygen

(2) *Platyhelminthes* —Both the species of turbellarian worm examined by us gave an unequivocal positive result as regards arginine phosphate Here again it may be questioned whether the main activity of the organism is not ciliary rather than muscular

(3) *Nemertinea* —Colorimetric comparison was rendered difficult in the case of *Lineus* by the presence of some substances in the extracts which tended to make them opaque and greenish However, as the amounts of phosphorus were much greater than in the case of the Cœlenterates, it was possible to obtain the values shown in Table II, demonstrating the presence of arginine phosphate

(4) *Annelida* — Of polychæte worms we had at our disposal *Sabellaria Spirographis* and *Nereis* The first two of these gave clear evidence of arginine phosphate, as the figures of Table III demonstrate, but otherwise there is little to say concerning them More thorough investigations were made on *Nereis* In particular we were able to show the functional significance of the phosphagen by experiments in which the animals were put into heat rigor, and stimulated to exhaustion in an atmosphere of nitrogen The latter operation was accomplished as follows a number of worms were placed in sea-water in a Buchner flask which was repeatedly exhausted by an electric Geryk pump and filled with purified nitrogen Stimulation was effected by a buzzer interrupter, induction coil and copper wire electrodes The duration of the stimulation was about 1 hour, at the end of which time the worms were

Table III —Direct Separation Method

Group.	Animal	Experiment No	Weight of material taken, gm	Mgm P			Mgm P/gm.				Phosphagen in per cent of the total P		
				A	B	C	A	B	C	D	B	D	Total
Annelids, Polychæta	*Sabellaria alveolata*— Whole body	AA 1	1 38	0 383	0 00	0 505	0 278	0 00	0 366	0 088	—	24 1	—
	,,	AA 2	2 45	0 550	0 00	0 789	0 224	0 00	0 321	0 097	—	30 2	—
	Spirographis brevispira— Whole body	R 1	2 83	0 281	0 00	0 768	0 099	0 00	0 271	0 172	—	63 5	—
	,,	R 2	3 08	0 301	0 00	0 925	0 098	0 00	0 300	0 202	—	67 0	—
	Nereis diversicolor— Whole body	L 1	2 53	0 530	0 10	0 865	0 209	0 04	0 341	0 092	11 7	27 0	38 7
	,	L 2	3 45	0 781	0 06	1 359	0 196	0 018	0 394	0 180	4 6	45 7	50 3
		L 3	2 80	0 588	0 07	1 040	0 210	0 025	0 372	0 137	6 7	37 1	43 8
	,	L 4	2 33	0 162	0 475	0 827	0 069	0 203	0 354	0 084	57 3	23 7	81 0
		L 5	2 18	0 470	0 00	0 769	0 215	0 00	0 352	0 137	0	37 0	37 0
	,	U 1	2 78	0 742	0 00	0 875	0 267	0 00	0 315	0 048	0	15 2	15 2
	,	U 2*a*	1 81	0 484	0 04	0 713	0 267	0 02	0 394	0 105	5 6	26 6	32 0
	, (heat rigor, 1 hour at 45° C)	U 3	1 59	0 888	0 00	1 058	0 558	0 00	0 666	0 108	0	16 2	16 2
	,, ,,	U 4	1 63	0 893	0 00	0 962	0 548	0 00	0 590	0 042	0	7 5	7 5
	,, (stimulated to exhaustion in N, see text)	U 5	2 47	0 095	0 00	0 935	0 362	0 00	0 378	0 016	0	4 2	4 2
Podaxonia	*Sipunculus nudus*— Body wall	Q 1	1 46	0 195	0 00	0 660	0 133	0 00	0 452	0 319	0	71 0	—

markedly unreactive, the whole bottle was then thoroughly cooled in ice before the worms were ground in ice-cold trichloracetic acid The following average results were obtained —

Conditions	Number of experiments	Arginine phosphate, mgm /gm	Arginine phosphate, per cent of total P
Normal	7	0 112	29 6
Heat rigor	2	0 075	11 8
Stimulation in nitrogen	1	0 016	4 2

The next question which arose concerned the meaning of the small amounts of phosphorus which appeared from time to time in the creatine phosphate fraction (B) from *Nereis* (experiments L 1, 2, 3 and 4, and U 2*a*) We were from the first inclined to regard these results as due to some special difficulty of separation in the extracts, the inorganic precipitate not being completely centrifuged off, and traces of it remaining suspended in the centrifugate This difficulty was particularly marked in L 4, while the separation was particularly good in L 5 We did not therefore accept the figures of fraction B as indicating the presence of creatine phosphate

However, in order to investigate the matter further, we carried out some experiments using the extrapolation method The results are shown in fig 1 and Table IV

In experiment U 1 a slight rise appeared during the progress of the readings on the colorimeter, and an apparent creatine phosphate value was thus obtained, although no creatine phosphate was found in this case by the direct separation method This led us, therefore, to regard the early rise in the curve with suspicion, and to think of it as perhaps due to the presence of some inhibitory substance which prevented the straightforward development of the colour It may be remembered that Fiske and Subbarow (1929) originally criticised the extrapolation technique (on which the Eggletons at first wholly relied) as subject to the interference of inhibitors, which would thus simulate labile phosphorus In experiment U 2*a*, the two methods agreed better, the one showing a small amount of phosphorus in fraction B, the other showing a slight rise with time But by the following experiment (U 2*b*) we were able to show that this was no evidence of creatine phosphate We let a portion of the original trichloracetic acid filtrate stand with molybdate for 20 minutes at room temperature, in order to decompose all the creatine phosphate, if any were present, and only after this began an extrapolation As is shown by Table IV and fig. 1, just the same result was obtained

Table IV

Experiment number	Direct separation, mgm P/gm			Extrapolation, mgm. P/gm.		
	A	B	A + B	A	B	A + B
U 1	0 267	0 00	*0 267*	0 178	0 039	*0 217*
U 2*a*	0 267	0 022	*0 289*	0 208	0 037	*0 245*
U 2*b*	0 267	0 022	*0 289*	0 221	0 026	*0 247*

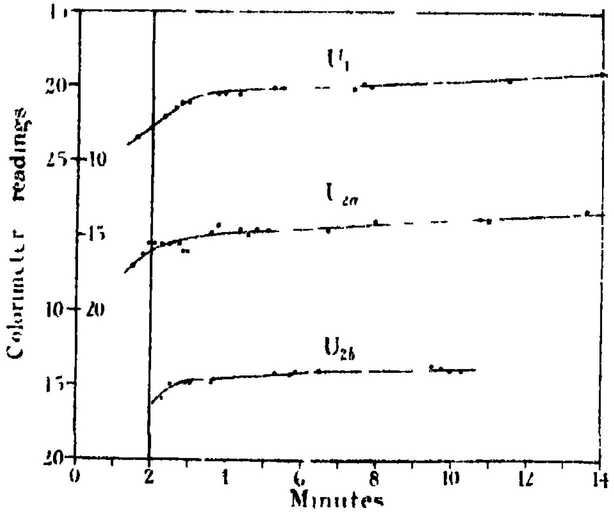

Fig 1 —Readings

	Initial.	Final
U 1	23 5	19 0
U 2*a*	16 0	13 5
U 2*b*	15 5	14 0

It is therefore probable that there exists in extracts of *Nereis* some substance which combines loosely with inorganic phosphate, preventing it from being fully precipitated by the ice-cold calcium method, and causing a slow development of colour when this is followed against a standard in the colorimeter There may also be something in the extracts which prevents the clean centrifugation of the precipitate of inorganic phosphate It is at any rate clear that in spite of the first appearances, polychaete worms contain arginine phosphate but not creatine phosphate.

This conclusion was further substantiated by the results of the Walpole technique In experiments L 3, U 5, L 5, and U 3 and 4, treatment with diacetyl gave only a yellow colour, quite distinct from the unmistakable pink given when creatine is present This was somewhat surprising as the test is believed to depend on the presence of a guanidine grouping, and the arginine due to the arginine phosphate should therefore have reacted However, it is probable that the workers who originally introduced the test (Vosges and Proskauer (1898)), and those who subsequently extended our knowledge of it (Harden and Norris (1911), Walpole (1911)) used much stronger concentrations of arginine than those with which we had to deal in our experiments Indeed, when we made up a series of standards from pure arginine, we found that although some trace of a gradation was visible between 0 05 and 1 0 mgm arginine in 4 c c the yellowish-orange colours were so faint that any quantitative comparisons were out of the question Yet at these dilutions, creatine gave pink colours which were quite strong enough for such comparisons How much arginine," said Walpole (1911 p 307) " is required to give the same colour as 1 mgm of creatine has not been determined ", from the above experiments it must evidently be a good deal more than 1 mgm O'Meara (1931) has recently reported difficulties in the use of the diacetyl reaction with arginine

Experiment L 5 filtrate was treated with copper-lime to remove the sugar, and then autoclaved before being estimated for creatinine by the Jaffe method An extremely slight reduction of the picric acid was observed, but in view of the well-known unspecificity of the Jaffe reaction, it is impossible to lay much emphasis on this

(5) *Podaxonia*—Our results for *Sipunculus* confirmed the statement of Meyerhof (1928) that this animal contains arginine phosphate The results are shown in Table III The Walpole reaction was negative and no creatine phosphate was found

(6) *Cephalopoda*—The mantle muscle of cephalopods is stated by Meyerhof (1928) in his paper on the Invertebrates to be free from phosphagen. We were, however, unable to confirm this finding, for as is shown by Table V, we constantly observed a rather small but nevertheless quite definite proportion of arginine phosphate in cephalopod muscle The only exceptions to this statement are two of the experiments in the AC series, but here it was noticeable that the animals were not in good condition, some were floating at the surface of the water in the aquarium, and the specimens chosen for analysis did not react by coughing, spitting, and ejecting ink, as usual upon capture As the

Table V —Direct Separation Method

Group	Animal	Experiment No	Weight of material taken, gm	Mgm. P			Mgm P/gm				Phosphagen in per cent of the total P
				A	B	C	A	B	C	D	
Mollusca Cephalopoda	*Sepia officinalis*—										
	Fin muscle	F 1	1 60	1 17	0 00	1 21	0 731	0 00	0 761	0 030	4 0
	Mantle muscle	F 2	3 43	3 70	Trace	4 25	1 520	Trace	1 750	0 230	13 2
	Funnel muscle	K 3	1 67	1 67	0 04	1 75	1 000	0 025	1 065	0 065	6 1
	Fin muscle	K 1	1 25	0 79	0 00	0 935	0 635	0 00	0 749	0 114	15 3
	Mantle muscle	K 2	1 19	2 50	Trace	2 73	2 100	Trace	2 290	0 190	8 3
	Funnel muscle	K 3	1 10	1 50	Trace	1 675	1 365	Trace	1 521	0 156	10 3
	Tentacle muscle	K 4	1 10	1 12	Trace	1 29	1 020	Trace	1 170	0 150	12 8
	Fin muscle	AC 1	0 76	0 64	0 00	0 69	0 837	0 00	0 908	0 071	7 8
	Mantle muscle	AC 2	0 40	0 805	0 00	0 78	2 010	0 00	1 950	0 00	0 0
	Funnel muscle	AC 3	0 56	0 747	0 00	0 73	1 330	0 00	1 300	0 00	0 0
	Tentacle muscle	AC 4	0 57	0 562	0 00	0 594	0 988	0 00	1 040	0 052	5 0
	Embryos ready to hatch (whole body)	A 1	2 58	0 535	0 00	0 719	0 206	0 00	0 277	0 071	25 6
	,, ,,	A 3	2 46	0 505	0 00	0 670	0 206	0 00	0 273	0 067	24 5
	Octopus vulgaris—										
	Mantle muscle	O 1	1 84	1 63	0 00	2 47	0 888	0 00	1 340	0 452	33 5
	Funnel muscle	O 2	1 53	0 73	0 00	1 005	0 477	0 00	0 659	0 182	27 7
	Tentacle muscle	O 3	1 66	1 42	0 12	1 77	0 855	0 07	1 065	0 135	12 7

animals in Meyerhof's tables are sometimes characterised by the phrase "*lang in aquarium*," we suggest that the presence of arginine phosphate can only be demonstrated in these animals if they are in perfect health, and freshly caught

Our manner of killing them consisted in conveying the animals direct from the aquarium into a large pan packed with ice, and after leaving them there for an hour, cutting sharply across the body with scissors just in front of the mantle opening

It will be observed in Table V that from time to time a trace of phosphorus appeared in the creatine fraction B We attribute this to the high concentration of inorganic phosphorus met with in these muscles

We had the opportunity of examining a considerable number of *Sepia* embryos at all stages of their development, but the full results on this subject are in course of publication elsewhere (Needham, Needham, Yudkin and Baldwin, 1932) We give two experiments for the sake of comparison in Table V *Octopus* muscle seems to show a higher arginine phosphate content than that of *Sepia*

When submitted to the diacetyl test for creatine, no trace of pink colour appeared in extracts of cephalopod muscle, a fact which substantiated the view expressed above regarding the figures in fraction B (experiments AC 1–4, O 1 and 2, F 2, K 1–4, and O 3) The Jaffe reaction was also completely negative after conversion by autoclaving (experiments O 2 and K 1–4)

(7) *Echinodermata* —In the two forms of Holothurian examined, arginine phosphate was only found in one, *Synapta*, but we do not regard the failure to find it in *Cucumaria* as significant, since a violent contraction of the body-wall muscles of this latter form occurred when it was being dissected, in spite of prolonged preliminary cooling in ice (see Table VI) Kutscher and Ackermann (1931) report the isolation of free arginine from *Holothuria tubulosa*

Greater interest attaches to the results obtained on the sea-urchin, *Strongylocentrotus lividus*, for the muscles of the jaw (Aristotle's lantern) yielded considerable amounts of labile phosphorus in fraction B as well as in fraction D Either creatine phosphate, or some phosphorus-yielding substance breaking down under the same conditions and at approximately the same rate, was present And from the outset it was obvious that the echinoid jaw muscles differed widely from any tissue so far examined, in that the amounts of arginine and creatine phosphate were of about the same order, amounting between

Table VI —Direct Separation Method

Group	Animal	Experiment No	Weight of material taken, gm	Mgm P			Mgm P/gm				Phosphagen in per cent of the total P		
				A	B	C	A	B	C	D	B	D	Total
Echinodermata, Holothuroidea	*Cucumaria planci*— Muscles of body wall	W 1	0 49	0 037	0 00	0 037	0 077	0 00	0 077	0 00	--	-	0 0
	,,	W 2	1 74	0 215	0 00	0 205	0 124	0 00	0 118	0 00	—	-	0 0
	Synapta inhaerens— Body wall	Y 1	0 90	0 315	0 00	0 425	0 350	0 00	0 472	0 122	—	25 9	25 9
	,,	Y 2	0 71	0 326	0 00	0 343	0 459	0 00	0 483	0 024		5 0	5 0
Echinodea	*Strongylocentrotus lividus*— Jaw muscles (Lantern of Aristotle)	I 1	1 01	0 214	0 088	0 405	0 212	0 087	0 400	0 101	21 5	25 2	46 7
	,, ,	I 2	2 98	0 514	0 331	1 090	0 172	0 111	0 366	0 083	30 3	22 7	53 0
	*, ,	I 3a	1 63	0 041	0 070	0 247	0 025	0 043	0 152	0 084	28 3	55 0	83 3
	*, ,	I 3b	1 63	0 020	0 062	0 247	0 012	0 038	0 152	0 102	25 0	67 0	92 0
	†, ,	I 4	1 41	0 169	0 139	0 690	0 120	0 098	0 490	0 272	20 0	55 5	75 5
	,	T 1	1 78	0 158	0 189	0 668	0 089	0 106	0 374	0 179	28 4	48 0	76 4
	,	T 2	1 85	0 161	0 213	0 745	0 087	0 142	0 402	0 173	35 4	43 0	78 4
	,, ,	AT 2	2 15	0 319	0 329	0 880	0 148	0 153	0 409	0 108	37 5	26 4	63 9
	, ,	BT	10 18	—	—	—	0 160	0 105	0 457	0 092	22 5	20 1	42 6
	(heat rigor, 1 hour at 45° C)	T 4	1 34	0 848	0 00	1 010	0 632	0 00	0 753	0 121	0 0	16 1	16 1
	, ,,	AT 1	2 05	0 486	0 055	0 114	0 288	0 033	0 675	0 354	4 8	52 7	57 5
	Eggs just fertilised	J 3	6 55	1 385	0 00	1 410	0 212	0 00	0 214	0 00	-	—	0 0
	Gastrulae	J 1	2 56	0 921	0 00	0 950	0 361	0 00	0 371	0 016	--	4 3	4 3
	,,	J 2	2 62	0 858	0 00	0 920	0 328	0 00	0 351	0 023	--	6 5	6 5
	Plutei	J 4	2 73	0 732	0 00	0 811	0 268	0 00	0 297	0 029		9 8	9 8
	,,	J 5	2 25	0 637	0 00	0 637	0 283	0 00	0 283	0 00		–	0 0
	Echinocardium cordatum— Spines and muscles	Z 1	2 55	0 027	0 00	0 027	0 011	0 00	0 011	0 00	—	—	0 0
	,,	Z 2	1 71	0 025	0 00	0 025	0 014	0 00	0 014	0 00	—	—	0 0
Asteroidea	*Asterias glacialis*— Tube-feet	N 1	3 84	0 072	0 00	0 308	0 019	0 00	0 080	0 061	—	76 2	76 2
	,,	N 2	4 17	0 094	0 00	0 338	0 022	0 00	0 081	0 059	—	73 0	73·0

* Indicates that the protractor muscles of the jaws and the depressor muscles of the compass were alone used

† Indicates that the retractor muscles of the jaws were alone used, in all the other cases the extraction was made on the whole mass of lantern muscles.

them to an average of 68 per cent of the total free and easily hydrolysable phosphate

A summary of the figures is given in Table VII

Table VII

	Experiment number	Per cent of the total free and easily hydrolysable water-soluble phosphate			
		Phosphagen			Inorganic
		Creatine	Arginine	Total	
Mixed jaw muscles, not cooled	I 1	21 5	25 2	46 7	53 3
Mixed jaw muscles, cooled	I 2	30 3	22 7	53 0	47 0
	T 1	28 4	48 0	76 4	23 6
	T 2	35 4	43 0	78 4	21 6
	AT 2	37 5	26 4	63 9	36 1
Protractors and Depressors	I 3	28 3	55 0	83 3	16 7
Retractors	I 4	20 0	55 5	75 5	24 5
Averages	-	28 7	39 4	78 1	31 8
Mixed jaw muscles, heat rigor	T 4	0 0	16 1	16 1	83 9
	AT 1	4 8	52 7	57 5	42 5
Averages	--	2 4	34 4	36 8	63 2

In this table there are several points of interest The first experiment made, in which the lanterns were not cooled before dissection, gave only about 45 per cent of the two phosphagens together instead of the 70 per cent which was found in later experiments after thorough cooling An attempt to decide whether the phosphagens were anatomically distributed, represented by experiments I 3 and I 4, showed that each kind of muscle has both phosphagens In heat rigor, the disappearance of the creatine phosphate was in one case complete and in the other case almost complete, on the two experiments the inorganic phosphate rose to 63 per cent instead of the usual 31 per cent

But the question clearly could not be allowed to rest at this point. If creatine phosphate were really present it was necessary to establish the fact as definitely as possible We therefore proceeded to a series of extrapolation experiments, which are summarised in figs 2, 3 and 4, and Table VIII In experiment T 1 the fresh trichloracetic acid extract of mixed jaw muscle was submitted to the extrapolation method, with results which agreed closely with those obtained by direct separation (see Table VIII) Experiment T 2

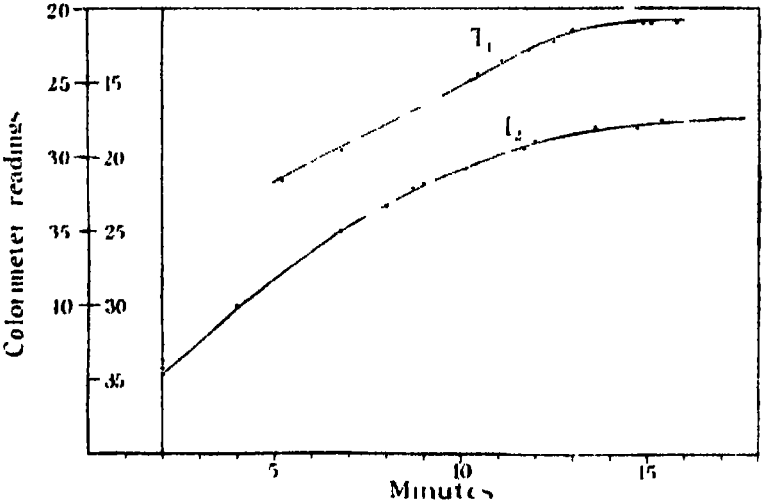

FIG 2

	Initial	Final
T 1	40 0	20 5
T 2	39 7	14 4

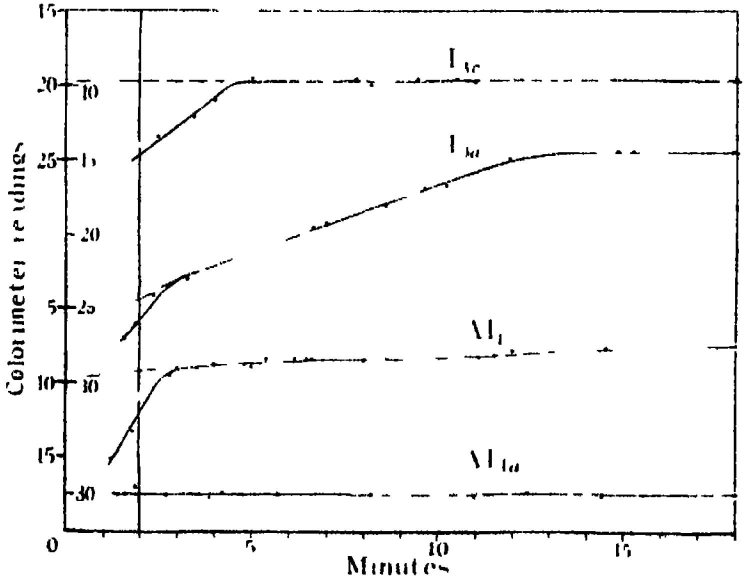

FIG 3

was a repetition of this, but so arranged that the colorimeter readings were begun not later than 2 minutes after the adding of the reagents Moreover, in order to avoid any effects due to unequal temperature, the standard was made up with 4 c c of distilled water previously cooled to 0° C in ice These findings demonstrate the presence of a labile phosphorus compound capable of rapid breakdown under the catalytic influence of molybdate

It was next decided to apply the extrapolation method to the fractions obtained by the separation method The inorganic fraction (A) should then give a perfectly straight line, and the creatine fraction (B) should give a marked rise These expectations were in the main fulfilled The curve in fig 3 marked T *3a* shows the result of following the development of the colour in untreated extract of experiment T 3, it is comparable with the curves of fig 2 The curve T 3c in the same figure is that given by the inorganic fraction when separated from the creatine phosphate fraction by precipitation with calcium The expected straight line is preceded by a rapid rise, due either to the presence of small amounts of (purple) echinochrome, leading to over-compensation on the part of the observer, or to the presence of an inhibiting substance We shall return to this question below The value indicated by the straight line agrees well with that found in the direct separation method (see Table VIII) As for the creatine phosphate fraction (B), the curve T *3d* in fig. 4 shows the result obtained after removal of the inorganic phosphate. The final value here again agrees with that found in the direct separation method

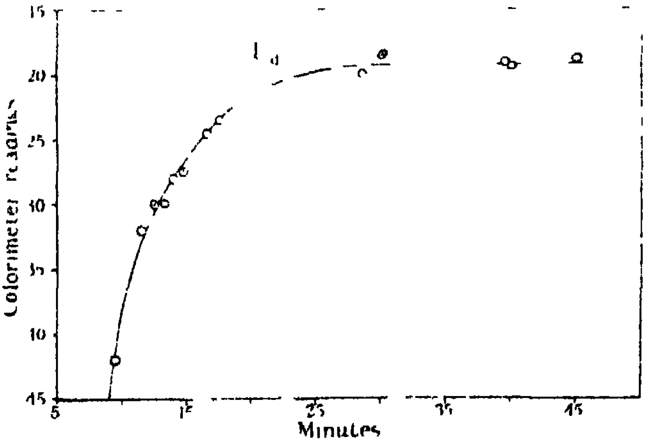

Fig 4

Another way of throwing light upon the situation was to submit the jaw muscles to heat rigor, in order to remove the creatine phosphate entirely or almost entirely, and then to carry out an extrapolation experiment on the extract There should in this case be practically no rise, and this was indeed the effect found by us in experiment AT 1, and shown in fig 3 and Table VIII The slight rise was of the order that would be expected from the amount of creatine phosphate disclosed by the direct separation method

Table VIII

Experiment number	Direct separation mgm P/gm			Extrapolation mgm P/gm		
	A	B	A + B	A	B	A + B
T 1	0 089	0 106	*0 195*	0 094	0 091	*0 185*
T 2	0 087	0 143	*0 229*	0 095	0 165	*0 260*
T 3*a*		–	–	0 158	0 134	*0 292*
T 3*c*		–	-	0 187	–	*0 329*
T 3*d*		–		–	0 142	
AT 1	0 237	0 027	*0 264*	0 285	0 013	*0 298*

In this case again, however, there was seen the rapid preliminary rise, ceasing before the end of the third minute after mixing If the very faint mauve tint of the extract, due to echinochrome were responsible for this, it would no doubt have its greatest effect in the early stages, when the blue colour was also faint But experiment AT 1*a* showed that it could not be responsible Some of the extract from experiment AT 1 was kept and incubated overnight as if for an estimation of total phosphate and this, next morning, was treated by the extrapolation method As appears from fig 3 the line was absolutely straight, although the echinochrome was still present The only remaining possibility was that the inhibitor already suggested had been destroyed on standing We had already found evidence of some substance which combined loosely with phosphate when considering the results of the work on *Nereis* The actual level of the straight line AT 1*a* corresponded to 0 537 mgm P/gm, *i e* (after subtracting the result of AT 1) 0 239 mgm P/gm arginine phosphate, as against the 0 293 mgm P/gm found by the direct separation method

From fig 4 it was possible to calculate a velocity constant for the hydrolysis of the labile compound in the presence of molybdate—the value obtained will be given below (p 280)

It was desirable, however, to obtain further information about the velocity constants of the hydrolysis of the two forms of labile phosphorus present in the echinoid jaw muscle The problem had never before arisen as this was the first known instance of the two phosphagens occurring side by side in the same muscle, but the means for solving it were presented by the following method

Muscle from the jaws of 16 well-cooled animals (10 18 gm) was extracted with trichloracetic acid in the usual way, filtration being performed with special precautions to keep the funnel cool The filtrate was brought to approximately decinormal acidity by the addition of 5 c c 40 per cent soda per 100 c c of filtrate, and at this point an aliquot portion (2 c c) was taken for the zero hour determination The whole filtrate was now brought to 28° C as rapidly as possible by immersion in a water-bath at about 33°, the time taken to reach 28° being noted The filtrate was then put into the electric incubator at 28° and samples, being withdrawn at suitable intervals up to 23 hours, were analysed for fractions A and B by the usual direct calcium separation method The essence of the procedure lay in the fact that as time went on the inorganic phosphate would be increased by a quota which would less and less be accounted for by the disappearance of the creatine phosphate This excess would be due to the hydrolysis of arginine phosphate, and the result would be a set of curves showing the total increase of phosphate, and the increase due to the respective phosphagens The data obtained in this experiment (BT) are shown in Table IX and fig 5

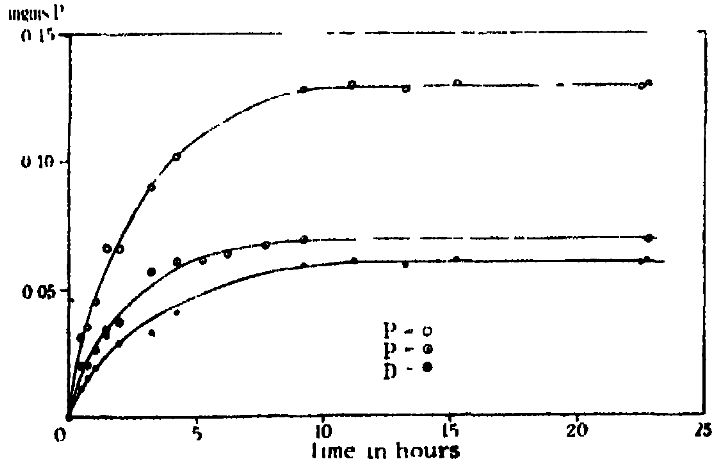

FIG 5 P = ○, P' ⊙, D ●

Table IX—Determination of Velocity Constants of Phosphagens in the Jaw Muscles of *Strongylocentrotus lividus* in 1 7 × N/100 Trichloracetic Acid at 28°

Time	Inorganic P A_t	Creatine P B_t	P $(A_t - A_0)$	P $(B_0 - B_t)$	D $(P - P')$	Remarks
hr min						
0 0	0 104(A_0)	0 069(B_0)	0 000	0 000	0 000	Daylight
0 10	0 1175	0 062	0 0135	0 007	0 007	
0 20	0 1175	0 060	0 014	0 009	0 005	
0 30	0 135	0 049	0 031	0 020	0 011	
0 45	0 139	0 049	0 035	0 020	0 015	
1 05	0 149	0 043	0 045	0 026	0 019	
1 30	0 170	0 035	0 066	0 034	0 032	
2 00	0 170	0 032	0 066	0 037	0 029	
3 15	0 194	0 012	0 090	0 057	0 033	
4 15	0 206	0 008	0 102	0 061	0 041	
5 15	0 206	0 008	0 096	0 061	0 035	Daylight lamp.
6 15	0 204	0 005	0 100	0 064	0 036	
7 45	0 206	0 000	0 102	0 067	0 035	
9 15	0 232	0 000	0 128	0 069	0 059	
11 15	0 235	Not estimated	0 130	0 069	0 061	
13 15	0 232		0 128	0 069	0 059	
15 15	0 235		0 130	0 069	0 061	Daylight
22 30	0 234		0 129	0 069	0 060	
22 45	0 235		0 130	0 069	0 061	
Final values	0 234	0 000	0 129	0 069	0 060	

Note — 2 c c samples treated with 2 5 c c molybdate and 1 c c sulphonic acid and made up to 25 c c At 5 h 15 m these quantities of reagents were insufficient, and after 7 h 45 m double quantities were used

A_0 = inorganic P present at beginning A_t — inorganic P present at successive times P = total increments of inorganic P B_0 — creatine P present at beginning B_t = creatine P present at successive times P′ = increments of inorganic P derived from creatine P D = increments of inorganic P derived from arginine P

From the data thus summarised, the velocity constants were calculated as follows —

	No of experiment	Temperature	Acid	Normality	Conditions	k 10^{-3}
Arginine phosphate	B1	28°	Trichloracetic	0 017 N	—	5·22
Creatine phosphate	BT	28°	Trichloracetic	0 017 N	—	5 81
	T 3*d*	18°	Hydrochloric	0 4 N	Molybdate present	115 0

The rates of breakdown of arginine phosphate and creatine phosphate from the echinoderm jaw muscles in deproteinated filtrate without molybdate, were thus very similar On comparing these results with those given in the literature, it was found that considerable confusion existed, some authors expressing their results in terms of $\log_e$ and others (*e g*, Meyerhof (1930) and Fiske and Subbarow (1929)) in terms of $\log_{10}$ As the former method is alone correct, we recalculated the data given for the phosphagens by previous observers, and constructed a composite chart which shows the relation between our results and theirs in terms of natural logarithms (fig 6) Meyerhof's figures (1928) for the velocity constant of hydrolysis of arginine phosphate show a maximum when the acid used is decinormal, and our value comes within his range of variation Similarly, allowing for temperature differences, our value for creatine phosphate agrees reasonably with those of Lohmann (1928) and of Fiske and Subbarow (1929) Moreover, the acceleration produced in the hydrolysis of our creatine phosphate fraction, when molybdate was present (experiment T 3*d*), shows convincingly that this labile compound in echinoderm muscle must be creatine phosphate At 18° (the temperature at which the hydrolysis of experiment T 3*d* was conducted) the velocity constant was 115 0, so that at 22° it would be about 161 0 But from fig 6 it may be seen that at this temperature the uncatalysed hydrolysis (from Fiske and Subbarow's results) would have a constant of 6 15, the increase due to molybdate was therefore one of 26 times This is in agreement with Meyerhof's finding (1928, p 538) that the hydrolysis, under such treatment, is accelerated some 30 times

Attention may now be directed to the results obtained by estimation of creatine (Walpole) and creatinine (Jaffe) The holothurian extracts were not kept for these methods, but we expected to have confirmation of the presence of creatine phosphate in the echinoid jaw muscles by using the diacetyl test In this, however, we were disappointed, nothing but a yellow colour being given with the extracts in experiments T 4, AT 1, and AT 2 Greater success was obtained by converting the creatine to creatinine and estimating it by reduction of picric acid Here two necessary controls were done (*a*) pure solutions of creatine were autoclaved parallel with the unknown solutions in order to check the degree of conversion, and (*b*) pure creatine solutions were treated by the van Slyke (1917) method for removing sugar, in order to check any possible loss Such loss, it was found, did not exceed 12 per cent

The results were as follows experiment AT 1 and AT 2 The filtrates were mixed, after the removal of sugar by copper precipitation, giving 9 8 c c, 4 c.c of which were autoclaved and estimated Of creatine 0 144 mgm were found (calculated from creatinine), *i e*, 0·36 mgm. as the degree of conversion

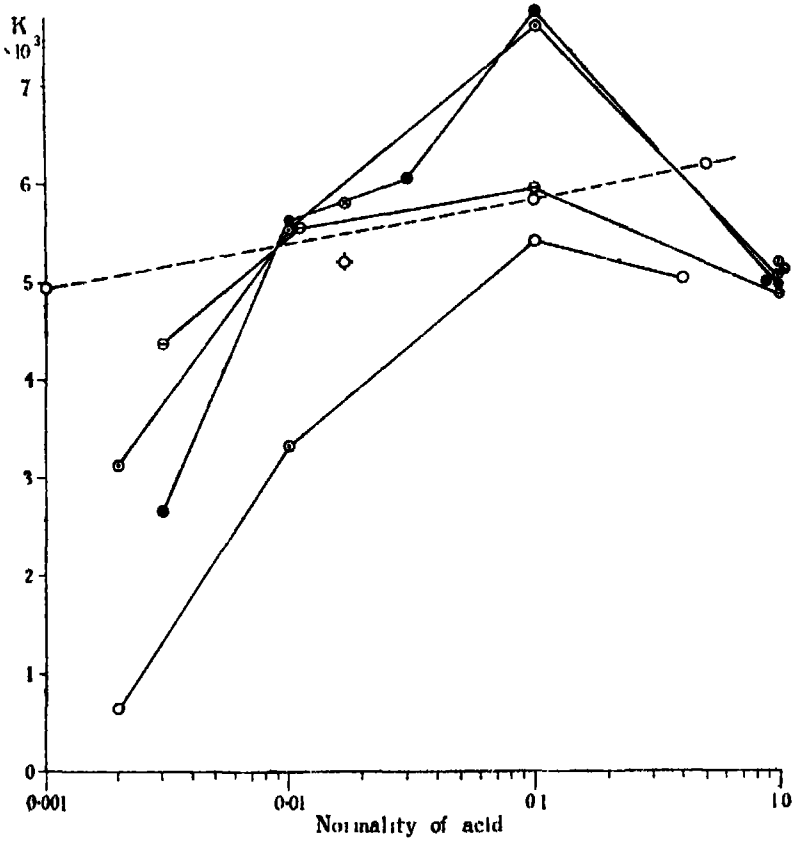

Fig. 6.—Semilog plot

Arginine Phosphate

Author	Animal	Temperature °C
Meyerhof (1928)	Mollusc	28 ⊙
"	Holothurian	28 ●
"	Podaxonian	28 ⊖
Meyerhof and Lohmann (1928)	Crayfish	28 ⦶
Meyerhof (1930)	?	28 ⦸
Present work	Sea urchin	28 -◇-

Creatine Phosphate.

Author	Animal	Temperature °C
Fiske and Subbarow (1929)	Cat. . .	22 ○
Lohmann (1928)	Frog	20 ⊕
Present work	Sea urchin	28 ⊗

was 40 per cent In the whole amount of filtrate (calculated from phosphate estimations) 0 78 mgm creatine was expected, or, in 4 c c, 0 32 mgm The correspondence was thus very close Similarly, in experiment T 2, 2·75 c c of filtrate were taken, prediction from the phosphate figures being 0 04 mgm. creatine per cubic centimetre, actual amount found 0·056 mgm creatine per cubic centimetre

In addition to the investigations of the jaw muscles, our intention originally was to undertake an ontogenetic study of the origin of phosphagen during echinoderm development The supply of ripe females, however, was so poor, owing to the lateness of the season, that we were only able to obtain the few figures shown in Table VI The collection of the eggs, the fertilisation, and the subsequent treatment of the material, were carried out as described by Needham and Needham (1930) From this it appears that eggs just fertilised contain no arginine phosphate, but that a very small amount is present in the gastrula and pluteus stages These amounts were so small that, in the absence of further experiments, we do not regard them as outside the limits of experimental error, but if the presence of phosphagen in echinoderm swimming larvæ should be confirmed, the association of phosphagen with ciliary activity, at which we have already hinted, will receive further support No evidence of creatine phosphate was found in the larvæ, and this was in agreement with the negative Walpole reaction given by experiment J 3 and the negative Jaffe reaction given by experiment J 4

The other echinoderms mentioned in Table VI are *Echinocardium* and *Asterias* We were not surprised that the former gave negative results, as the only part of the animal suitable for our analysis was the shell, including the silky spines and the muscles which operate them The tube-feet of the starfish, however, gave considerable amounts of arginine phosphate, but owing to the difficulties already referred to in the case of *Sepia*, no confirmation of this by the Walpole method was obtainable

It appears to us that the identification of creatine phosphate in echinoderm muscle is the best evidence which has so far been brought forward in favour of the presence of creatine in this phylum Other investigators claim to have found small amounts in echinoderm blood (Myers, 1920) and cœlomic fluid (Heyde, 1923), but their observations were wholly dependent on the unspecific Jaffe reaction

(8) *Hemichorda*—Unlike all the other animals studied, *Balanoglossus salmoneus* was not available in the immediate neighbourhood of Roscoff, so an expedition was made, on the occasion of a low spring tide, to Morgat, where

some specimens were obtained These were conveyed to the laboratory as quickly as possible in cool damp sand, and estimations were begun immediately upon their arrival The animals, which were giving off their usual strong odour of iodoform (*cf* Bateson, 1885, p 91) were in an actively contractile state before being cooled prior to grinding in trichloracetic acid Table X gives the results obtained Material from the proboscis region (experiment X 1) gave clear evidence both of creatine and arginine phosphate, but that from the gill region, in experiment X 2, showed no arginine phosphate The other experiments were not quite so complete, but X 3 confirmed the two previous ones as regards the amount of creatine phosphate A portion of trunk region, instead of being worked up immediately, was left 24 hours (until moribund and hardly contractile) and then estimated (experiment X 7), with the result that all the creatine phosphate and some of the arginine phosphate was found to have disappeared This was of much interest in that it confirmed an experiment made earlier in the summer by Dr John Runnstrom on a piece of *Balanoglossus* which had been collected the day before and left over from some zoological researches Estimating the labile phosphorus, he found no creatine phosphate and about 10 per cent of arginine phosphate It is clear, therefore, that in order to demonstrate the presence of creatine phosphate in hemichord tissue it is necessary to operate on material as soon as possible after its removal from its natural surroundings We would here express our acknowledgments to Dr Runnstrom for kindly allowing us to refer to his unpublished observation

We were able to confirm, by the aid of the extrapolation method, the presence of creatine phosphate, or of some labile phosphorus compound breaking down at the same rate and under the same conditions The results of two such experiments are shown in fig 7 and the comparisons with the direct separation method in Table XI (see p 286)

There can be little doubt but that in this case the direct separation method is the more accurate, the constantly higher figures of the extrapolation method being due to the difficulty of matching the blue colours in the presence of small amounts of the natural pigment of the animal Although the animal itself is a bright yellow, the sea-water in which it has remained for some time is coloured bright pink, and it was some of this pink pigment which appeared in our extracts However, the graphs of fig 7 give valuable support to our first conclusion that *Balanoglossus salmoneus* contains creatine phosphate

Still further support was afforded by the results of Walpole and Jaffe estimations With the diacetyl treatment experiment X 6 (1 6 gm of trunk region in 15 c c trichloracetic acid) gave the pink colour so characteristic of

Table X.—Direct Separation Method

Group	Animal	Experiment No	Weight of material taken, gm	Mgm P			Mgm P/gm				Phosphagen in per cent of the total P		
				A	B	C	A	B	C	D	B	D	Total
Hemichorda	*Balanoglossus salmoneus*—												
	Proboscis and collar region	X 1	0 36	0 057	0 021	0 101	0 160	0 060	0 280	0 060	21 4	21 4	42 8
	Region of gills	X 2	1 3	0 293	0 051	0 349	0 225	0 039	0 268	0 00	14 5	0 0	14 5
	Trunk	X 3	1 6	Lost	0 124	0 226	Lost	0 077	0 142	’	21 1	—	—
	Region of gills	X 5	1 13	0 188	0 188	—	0 166	0 166	—	—	—	—	—
	Trunk (after 24 hours)	X 7	0 6	0 075	0 00	0 090	0 125	0 00	0 150	0 025	0 0	16 6	16 6
Urochorda	*Ascidia mentula*—												
	Muscle sheets of atrial cavity	H 1	7 03	0 039	0 00	0 050	0 005	0 00	0 007	0 0016	0 0	22 5	22 5
	Sphincter muscles of mouth and atrial aperture	H 2	3 32	0 080	0 00	0 092	0 024	0 00	0 028	0 0035	0 0	12 5	12 5

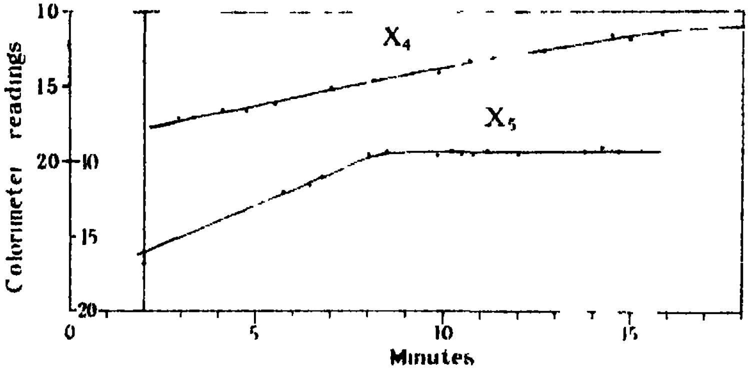

Fig 7

	Initial	Final
X 4	18 7	11 0
X 5	18 0	9 3

Table XI

Experiment and number	Direct separation, mgm P/gm			Extrapolation, mgm. P/gm		
	A	B	A + B	A	B	A + B
Trunk—						
X 3	—	0 077	—	—	—	—
X 4	—	—	—	0 225	0 137	0 36
Gill region—						
X 5	0 166	0 166	0 332	0 211	0 213	0 424

creatine, and it was easily possible to estimate the amount present by comparison with a series of standards No estimation of creatine phosphate in X 6 had been made, but the average value for creatine phosphate in milligrams per gram was found to be 0 11

Experiment number	Creatine P, mgm /gm
X 1	0 06
X 2	0 04
X 3	0 07
X 4	0 14
X 5	0 21
	0 16
Average	0 11

Taking this figure as probable for X 6, 0 17 mgm creatine phosphate P should have been present in the extract, *i e*, 0 68 mgm creatine The actual amounts found in two estimations were 1 10 and 0 90 mgm respectively

A similar close correspondence was found when the creatine was converted to creatinine and estimated by the reduction of picric acid The remainders of the extracts of experiments X 1–5 were massed together, making 14 c c , this was evaporated to 3 75 c c and any sugar removed by van Slyke's method (1917) The result was 0 485 mgm creatine (calculated from the creatinine), or, allowing for the fact that 60 per cent of a pure solution of creatine autoclaved at the same time was converted to creatinine, 0 82 mgm In experiment X 6, 1 5 c c were similarly treated, yielding, when treated by the conversion factor 0 186 mgm or, in 15 c c 1 86 mgm Another sample of 4 c c gave 0 238 mgm creatine, or, in 15 c c 0 91 mgm

Summarising—

	mgm
Creatine expected assuming the average of all the estimations as the value for X 6	0 68
Creatine found—	
By estimation of creatine (Walpole)	1 10
	0 90
By estimation of creatinine (Jaffe)	0 82
	1 86
	0 91

In view of the fact that the organism probably contains some free creatine, this agreement may be regarded as satisfactory

(9) *Urochorda*—The tunicates, as degenerate vertebrates only possessing a notochord in the larval state, were of much interest from our point of view. Unfortunately, it was impossible to have the ascidian "tadpoles" in sufficient numbers for chemical analysis, owing to the difficulty of obtaining sufficient eggs and the problem of mass culture We think it probable, however, that in those parts of the world where tunicates which "incubate" their larvæ in the atrial cavity (*e g*, *Corella*) are available, a sufficient mass of tadpoles might be obtained from one or two animals

The outstanding difficulty in dealing with the tunicates we found to be the very watery character of their tissues, and the extremely small amounts of phosphorus contained in them Thus in Table X it can be seen that from as much as 7 gm of atrial muscle, we only obtained 0 05 mgm of total inorganic and labile water-soluble phosphorus The figures for arginine phosphate, therefore, depend only on extremely small differences, and we are not prepared to lay great emphasis on them in the absence of further experiments. Anticipating that the Walpole method would estimate arginine in such small concentrations, we took 48·8 gm of atrial muscle sheets (corresponding to several hundred animals) and extracted it with 43 c c of 4 per cent trichloracetic acid (experiment H 3) But in due course we found, as has already been said, that arginine in small amounts cannot be estimated in this way A positive Walpole reaction for creatine, however, was not seen When some of the extract was treated with copper-lime to remove sugar, and then, after autoclaving, estimated for creatinine by the Jaffe method, a small quantity of some substance reducing picric acid, was observed This amounted to 0 44 mgm in the *whole* of the extract from 49 gm of muscle—a quantity, which if present originally as creatine phosphate, could not have been detected by our methods But we hesitate to accept this evidence as indicating creatine phosphate in ascidian tissue, in view of the many other substances which reduce picric acid

The position is then very unsatisfactory, and until further experiments can be undertaken, in which large amounts of ascidian tissue can be worked up for creatine or arginine, no further information will be available

Discussion

The importance of phosphagen in muscular contraction has already been referred to in the Introduction to this paper, but one result of the comparative investigations here reported has been to raise the question of whether ciliary activity may not also involve the phosphagen mechanism In certain of the organisms examined, especially the ctenophores and the echinoderm larvæ, ciliary activity is, at any rate at first sight, a much more characteristic function than muscular activity, and the association here suggested would be worth extended examination

The energy-providing function of phosphagen breakdown is clear in those cases—only the frog and crayfish—where the question has been specifically investigated That the same conception may be extended to other muscles seems probable from the numerous other cases where arginine or creatine phosphate has been shown to break down on stimulation or rigor (*Sipunculus*, *Holothuria*, *Pecten* (Meyerhof, 1928), *Raia clavata* (Eggleton and Eggleton, 1928); *Nereis*, *Strongylocentrotus*, *Balanoglossus* (the present work), frog heart (Eggleton, Eggleton and Clark, 1930). But the correlation between the type of contraction and (*a*) the type of phosphagen present, (*b*) the amount of phosphagen present, (*c*) the rate of its breakdown, is at present obscure. *In vitro* at p_H 7·0 arginine phosphate breaks down more slowly than creatine phosphate, and, per molecule of H_3PO_4, provides rather less energy. Ritchie (1928) has shown how, in slow tonic contractions which may be regarded as built up of a number of slow twitches, the energy needed for long-continued contraction is small. In many invertebrates contraction is of this kind and the amount of phosphagen present is small, as is also the lactic acid formation. In other invertebrate animals, *e.g.*, *Nereis*, *Octopus*, the movements are rapid and energetic, yet these animals also contain only the arginine phosphate, and that in rather small quantity. Similarly, Boyland (1928) has found that *Sepia* muscle shows very little lactic acid formation *in vitro*. The involuntary muscle of vertebrates, such as that of the frog's stomach, having very much the same type of movement as *e.g.*, *Sipunculus*, contains only creatine phosphate (Needham and Baldwin, 1932)—and arginine phosphate is absent from the heart and stomach of rat and frog. It seems at present an open question whether the appearance of creatine phosphate in the higher animals was an evolutionary change of advantage to them, or whether it was merely a change of no moment in itself, following of necessity in the train of some other mutation. It would not be unreasonable to regard arginine phosphate *a priori* as the more "primitive" of the phosphagens, in view of the fact that a linkage between the terminal arginine of the protein and the phosphoric acid of the nuclein probably occurs in the nucleoprotein molecules essential in every cell (*cf.* Waldschmidt-Leitz, Ziegler, Schaffner and Weil, 1931).

We may now turn to the phylogenetic aspect of the work. The investigations reported in this paper have led to a considerable extension of the data previously available concerning the distribution of phosphagens in invertebrates. They may conveniently be summarised as follows, according to the type of compound found:—

No Arginine phosphate—		
Cœlenterata	*Anthea*	Tentacles and body-wall
Arginine phosphate alone—		
Cœlenterata	*Pleurobrachia*	Whole body
Platyhelminthes	*Planaria*	,,
	Polycelis .	,,
Nemertinea .	*Lineus* .	,,
Annelida	*Sabellaria* .	,,
	Spirographis	,,
	Nereis .	,,

Arginine phosphate alone (contd.)—

Podaxonia	*Sipunculus*	Body-wall
Cephalopoda	*Sepia*	Fin, funnel, mantle and tentacle muscle
	Octopus	,, ,,
Echinodermata	*Synapta*	Body-wall
	Asterias	Tube-feet
Urochorda	*Ascidia*	Muscle

Creatine phosphate and arginine phosphate together—

Echinodermata	*Strongylocentrotus*	Jaw muscle
Hemichorda	*Balanoglossus*	Whole body

For the sake of completeness, there may be added, from the previous work of other authors --

Creatine phosphate alone—

Cephalochorda	*Amphioxus*	Whole body
Craniata	Many different species	

It may thus be seen that alone among the Invertebrates, the Echinoderms have given evidence of creatine phosphate, and that among the Protochordata, both Hemichorda and Cephalochorda contain creatine phosphate. But while *Amphioxus* contains creatine phosphate alone, and thus allies itself with the whole of the Craniata, *Balanoglossus* appears to contain both creatine and arginine phosphate, and thus allies itself with the Echinoderms, as represented in our researches by the echinoid jaw muscles. These facts acquire particular interest in view of the central position which *Balanoglossus* occupies in the phylogenetic affinities of the vertebrate Phylum.

It is to Kovalevski (1866) that we owe the first description of the anatomy of an enteropneust, for in 1866 he published a fairly correct account of some specimens of *Balanoglossus*. But it was not until the classical series of papers by Bateson from 1884 to 1886 that the foundations of our embryological and morphological knowledge of the organism were laid (1884, 1885, 1886, *a*, *b*). The most important point which he elaborated was that *Balanoglossus* possesses at one and the same time a notochord (in the adult state) and a larval form strongly resembling those of echinoderms. The larval form ("Tornaria") had indeed been described much earlier than the adult form (by J. Müller (1849)), and had been regarded as a variety of echinoderm bipennaria larva, differing however in possessing eye-spots and a peri-anal row of cilia. It was not until 1881 that Metschnikov (1881) discovered the real destiny of the tornaria larva, and as at that time the larva was better known than the adult, he proposed to make a new division of echinodermata, the "Bilateralia," the remainder of the group forming a parallel division, the "Radiata" But this suggestion, based as it was almost wholly on the larval characteristics, could serve no better than the old designation of the adults as ' worms," and after Bateson's (1884) brilliant demonstration of the existence of a notochord in the adult, the situation could only be met by making the Enteropneusts into a separate group, having affinities with the Chordates on the one hand and with the Echinoderms on the other "We must assume," said Bateson (1886, *b*, p 565) "some affinity between Echinodermata and Chordata, for Tornaria is not merely very like, but practically identical with, Bipennaria"

These results were challenged by Spengel (1894) In his monograph on the Enteropneusts in 1894, Spengel denied all relationship to the Chordata and suggested distant annelid affinities, regarding Tornaria as a very aberrant trochosphere His conclusions however, were successfully attacked by MacBride (1894), who almost exactly 20 years afterwards could write (1914, p 584) "Spengel s criticisms of Bateson's results, based on his own observations on Tornaria larvæ, made many years ago by methods which are now superseded, are no longer valid, as he himself would be the first to admit"

The results reported in the present paper seem to offer, from an unexpected biochemical quarter, some evidence in favour of Bateson's view If the association between creatine phosphate and vertebrate morphology may be regarded as established, so that, in Meyerhof's phrase, a "chemical mutation" in phosphagen construction occurs at the vertebrate level, then it can hardly be without significance that alone among all the invertebrate tissues examined, echinoid muscle turns out to possess what would otherwise be a prerogative of the Chordata, including *Balanoglossus* The Echinoidea are generally regarded as the most specialised, complicated and temporally recent, of the echinoderms, and it might therefore be possible to say that the most primitive echinoderms possessed the potentiality of developing creatine phosphate, but that this did not actually occur until the advent of the echinoids with their exceedingly complicated and specialised jaw muscles From this point of view, we were disappointed in not being able to examine any Crinoids, as representing the oldest living types of echinoderm

It might, of course, be argued that the presence of creatine phosphate in echinoderm muscle was primarily due to the fact that these jaw muscles are striated. This would avoid bringing in any evolutionary considerations But a strict association between creatine phosphate and striated muscle in the invertebrates cannot be maintained in view of the negative findings of the Eggletons and Meyerhof on the striped adductor muscles of molluscs, and the voluntary muscles of crustacea

The Echinoderms are not the only invertebrate group which has been suggested in connection with the origin of Vertebrates On account of the metameric repetition of organs, Dohrn (1875) and Semper (1876) regarded the Annelids as the most promising Phylum from this point of view, while Balfour (1881) in the second volume of his great work "Comparative Embryology," suggested that the Nemertines, with their two lateral nerve-cords, might indicate the direction whence the Vertebrates had come This theory was further elaborated by Hubrecht (1880, 1887) Then Gaskell (1908), in a work which never received general approval, urged the claims of the Arthropods, believing that the central canal of the Vertebrate spinal cord represented the invertebrate gut, and that a new intestinal tract had originated *de novo* on the ventral surface Could it be said that the biochemical results reported in this paper, uniting as they do Echinoderms, Enteropneusts, and Vertebrates, suggest that these and other similar lines of thought were misguided, and that the tradition associated with the names of Bateson, MacBride and Garstang is right?

At present we must content ourselves with the mere statement of this question

Summary

(1) A systematic survey has been made of the phosphagens of Invertebrates

(2) Arginine phosphate exists in all the Phyla of which representatives were studied, but among the Cœlenterates it could only be demonstrated in a Ctenophore The velocity constant obtained for its hydrolysis agreed with the values of previous workers

(3) Other evidence is also brought forward which suggests that arginine phosphate may be associated with ciliary as well as with muscular movement

(4) Contrary to common belief, creatine phosphate is not confined to the Vertebrates, but was found to be present (*a*) in Echinoderm jaw muscle (*Strongylocentrotus*), and (*b*) in Enteropneust tissues (*Balanoglossus*). Creatine phosphate was identified (i) by the direct estimation method (calcium precipitation, Fiske and Subbarow), (ii) by the extrapolation method (Eggleton and Eggleton), (iii) by the determination of the velocity constant of its hydrolysis in the presence and absence of molybdate, (iv) by estimation of the creatine

(Walpole), and (v) by conversion of the creatine to creatinine and estimation of the latter (Jaffe)

(5) If any evolutionary significance may be attached to these findings, it is probable that they support the Echinoderm-Enteropneust theory of Vertebrate descent (Bateson, MacBride, Garstang), rather than any of the other views which have from time to time been put forward on this question

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The Reproductive Processes of certain Mammals. III. *The Reproductive Cycle of the Male Ferret*

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(Communicated by C Lovatt Evans, F R S —Received December 23, 1931)

[Plates 10-12]

I *Introduction*

Cyclic changes in the reproductive organs of male mammals have been studied in a few forms only, but the information available reveals interesting differences in the duration of reproductive activity, and in the correlation between the various organs Marshall (1911) has described the reproductive cycle in the male hedgehog Regaud (1904), Tandler and Grosz (1911) and Lecaillon (1909) have studied the mole, but with conflicting results Rasmussen (1917 and 1918) gives detailed information of the periodic changes in the interstitial tissue of the testis in the woodchuck, and more recently Courrier (1923) has investigated the reproductive cycle in various bats in the mole, hedgehog, and marmot

The work of Marshall (1904), Robinson (1918) and Hammond and Marshall (1930) has established that in the female ferret the breeding season is restricted and lasts from April to August , during the remaining months of the year the reproductive organs are in a state of quiescent anœstrus The first mating occurs in March or April The present investigation was undertaken in order to determine the duration of the quiescent period in the male ferret and to compare the changes in the testis tubules with those in the interstitial tissue and accessory organs The condition of the reproductive tract has accordingly been studied at various times of the year, and an attempt has been made to assess the changes in the organs and in their constituent parts

II *Material and Technique*

Source of Animals —The 16 adult animals used in this investigation were ferrets or so-called pole-cat crosses obtained from dealers. They were kept in the laboratory for at least 2 or 3 weeks before killing, to avoid variation in the state of the reproductive organs due to feeding or other conditions

The weight and general condition of all the anœstrus animals except one (F 10) showed them to be at least second year adults, so that the anœstrus quiescence cannot have been due to the animals being immature from the previous breeding season

In F 8, the left testis and epididymis were removed in February, the right testis, epididymis, and vas deferens in June, thus seventeen stages of the testis cycle are available The three immature animals were bred at University College, London, and killed at the age of 2 months (F.72), 4 months (F 15), and 5 months (F 16)

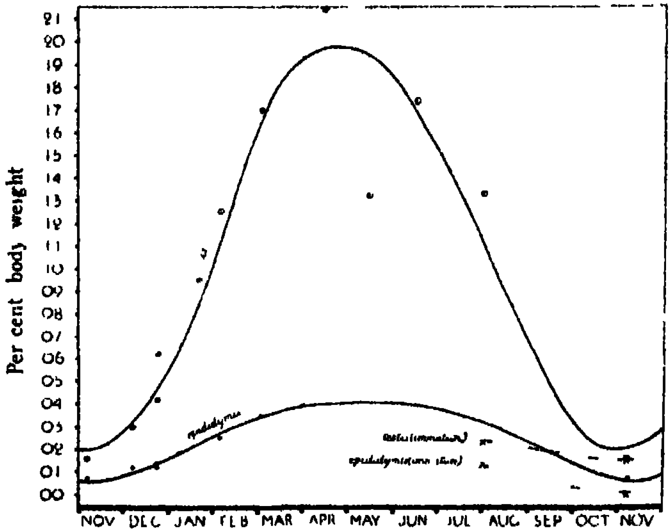
The testes in the ferret remain in the scrotal sacs throughout the year As in many carnivores, accessory sexual glands are vestigial

Histological Technique —The animals were killed with chloroform, the testes, epididymes, vasa deferentia, and penis dissected free from connective tissue and fat, and fixed immediately in Bouin's fluid (alcoholic), with the exception of a small piece of the left testis which was fixed in Flemming's strong fluid The organs were upgraded to 70 per cent alcohol and then weighed on a torsion balance having an accuracy of ± 0 5 mgm The testis weight given below is the average weight of the two testes, or the weight of the right testis where the left was used for fixation in Flemming's fluid Little asymmetry in weight was observed The epididymis weight was arrived at in the same way Sections of the Bouin material were cut at 7 μ and stained in Ehrlich's hæmatoxylin and eosin, and also in iron hæmatoxylin Mallory's triple connective tissue stain was found useful for the study of the interstitial cells, and in estimating the variation in the amount of fibrous tissue and muscle tissue in the epididymis and vas deferens Sections of the Flemming-fixed testis were cut at 5 μ and mounted unstained in Farrant's glycerine medium to study the changes in the fat content of the tissues

In each animal the diameter of the seminiferous tubules was obtained from camera lucida drawings made from an approximately median transverse section Fifty tubules were measured, the least diameters being taken in order to avoid apparent increase due to coiling of the tubules Tubules from the periphery were found most suitable owing to the difficulty of fixing the larger testes Since the tubules selected in all animals were situated in the same area, a zone extending three tubules deep round the periphery, these measurements can legitimately be used for comparative purposes

III *Changes in the Testis*

(*a*) *Gross Size* —The weight of the testis was found to vary from 0 178 gm. in November to 2·062 gm in April (see Table I) Calculated as a percentage of the body weight, the testis thus varies from 0·016 per cent to 0·219 per cent In Table I are given the data from 15 adult animals and 3 immature animals. Text-fig. 1 shows the variation in testis weight as a percentage of the body weight during the year. It will be seen that the figures rise steeply and regularly from November to April and show comparatively little individual variation The percentage weights in May, June, and August are considerably below the figure reached at the beginning of the breeding season, and it is probable that the testis diminishes in size throughout the summer months, although spermatogenesis remains active until August There is more individual variation at this time of the year



TEXT-FIG 1 —Variation in weight of testis and epididymis

In the immature animals, the 2 months old animal killed in August has a testis weight of 0·034 gm, or 0·023 per cent body weight, while the 5 months animal killed in November has a testis weight of 0·087 gm, or 0·016 per cent. body weight, *i e*, though the body weight increased nearly four times, the percentage testis weight has decreased.

The increased size of the testis in the breeding season is due to increase in—

(i) the diameter of the testis tubules, due to proliferation of the germinal epithelium;

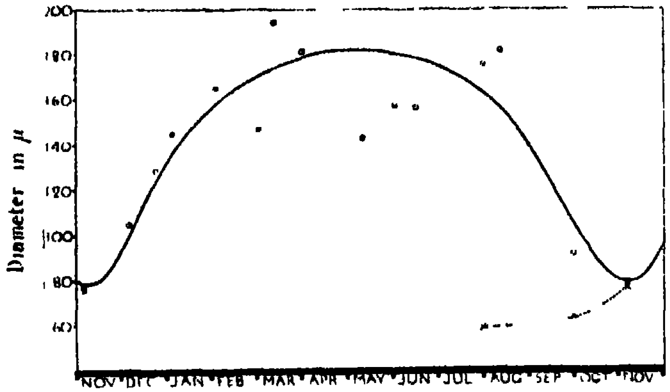
(ii) the length and convolution of the tubules, so that a median cross section of the testis in the winter months contains fewer tubules than a section in the same region of the testis during the breeding season;

(iii) the size of the interstitial cells,

(iv) the blood supply—in the quiescent testis it is extremely difficult to detect any capillaries in the intertubular spaces, but in the breeding season they are numerous and gorged with blood

(*b*) *Diameter of the Seminiferous Tubules*—The increased diameter of the testis tubules in the breeding season has been noted in the hedgehog, mole, and bat But Rasmussen (1917) states that in the woodchuck there is little cyclic variation in the size of the tubule , all increase in size of the testis is due to enlargement and proliferation of the interstitial tissue

It was thought desirable, owing to the conflicting opinions expressed by other workers on the variation in amount of the interstitial tissue, to measure the seminiferous tubules throughout the cycle in order to ascertain how far the apparent increase of interstitial tissue in the winter testis might be due to collapse of the testis tubules, and consequent enlargement of the intertubular spaces In Table I are given the average diameters of the testis tubules of adult and immature animals, and in text-fig 2 these figures are plotted against the month of the year



TEXT-FIG 2 —Seasonal variation in diameter of testis tubules

The average diameter of the tubules varies from 70–80 μ in October and November to 150–190 μ in the breeding season As with the weights of the testis, the tubule figures for November to April rise gradually to a highest point at the beginning of the breeding season From May to August the average diameter varies from 140–180 μ, and it is still high in the middle of August There is probably a gradual decrease in diameter throughout September, since by the beginning of October the tubules have diminished to 90 μ in diameter figs 1 and 2, Plate 10 The tubules in the prepubertal animal increase from an average diameter of 58 μ at 2 months old to 76 μ at 5 months old, when they approximate closely in size to the tubules of the adult at the same period

(*c*) *Spermatogenesis*—The changes in diameter of the testis tubules are correlated closely with variation in the activity of the germinal epithelium

In October and November when the diameter of the tubule is least, the testis as a whole has a prepubertal appearance, fig 1, Plate 10 The majority of the tubules are lined by a single layer of cells consisting of the nuclei of Sertoli and occasional spermatogonia. The Sertoli nuclei stain darkly, and are oval and smooth in outline, their chromatin is in the form of coarse masses The cytoplasmic syncytium of the Sertoli cells entirely fills the tubule, fig. 5, Plate 11 The spermatogonia, which are far fewer in number than the Sertoli nuclei, are large, lightly staining cells with their cytoplasm definitely delimited from the Sertoli syncytium Their nuclei are spherical and much lighter than those of the Sertoli cells Some of these spermatogonia are relatively enormous cells, the "winter spermatogonia" described by van Beneden (quoted by Courrier, 1923, *b*) in the bat, and by Courrier in the bat, mole, marmot and hedgehog, fig. 5, Plate 11 Courrier, from his observations on bats, claims that these "winter spermatogonia" arise by hypertrophy of the smaller summer spermatogonia, but it is difficult to follow this transformation in the ferret owing to the shorter duration of the quiescent condition Since they are present in 5 months old animal, however, it seems probable that they represent the normal condition of the resting spermatogonia In a number of tubules in the October and November testes, primary spermatocytes in diakenesis are present, but no later stages of spermatogenesis Occasionally masses of degenerating spermatids or spermatocytes occur in the October testis, but these were not found during November

At the beginning of December (F 18), signs of activity reappear The tubules contain primary spermatocytes arranged regularly two or three rows deep and not scattered as during November The Sertoli nuclei, which in the more quiescent testis were crowded together in uneven rows, have now retreated to the connective tissue sheath "Winter spermatogonia" are still present but are fewer in number, and there are numbers of a new generation of spermatogonia These are smaller cells with a coarser reticulum of chromatin in the nucleus, fig. 6, Plate 11

By the end of December the tubules have two or three rows of primary spermatocytes and an obvious lumen These are clearly a new generation of primary spermatocytes, since their nuclei are nearly all in synapsis, whereas during October and November the nuclei of the primary spermatocytes are in diakenesis In F.21 killed at the beginning of January many of the primary spermatocytes are dividing to form secondary spermatocytes These appear to degenerate, the lumen of most of the tubules contains masses of spermatocytes, primary and secondary, which have broken loose from the germinal epithelium The degenerate masses are also seen in the tube of the epididymis In F 10, killed in the third week of January, the condition of the germinal epithelium is very similar "Winter gonia" are no longer present, the spermatogonia have smaller nuclei and their cytoplasm is merged in the syncytium of the tubules The Sertoli nuclei are now greatly enlarged and are lightly staining They appear fewer than in the quiescent testis, but this is probably due to their scattering in the larger tubules

F 22, killed at the beginning of February, shows a great advance in the germinal epithelium There are several rows of spermatids, which are already elongating to form spermatozoa in many tubules Very occasionally fully formed spermatozoa are seen, fig 7, Plate 11. By the beginning of March the tubules are actively producing sperms, and they continue to do so throughout the summer months, fig 2, Plate 10

In the August testis there are signs of regression and more variation in the condition of adjacent tubules than at any other period of the year In many the lumen is wide and contains masses of spermatids and spermatocytes separated from the germinal epithelium, fig 8, Plate 11 During August and September there must be a rapid regression of the germinal epithelium, since by the beginning of October the tubules show no stages later than primary spermatocytes, and have contracted in diameter from 170–180 μ to 90–100 μ

The three immature animals show various stages in pre-spermatogenesis. In F 72, killed at the age of 2 months, the tubules are lined with one layer of cells, the gonia and Sertoli nuclei The cytoplasm of the Sertoli cells is drawn out into processes stretching across the lumen Occasionally the spermatogonia are seen in the lumen of the tubule In the 4 months old animal killed at the beginning of October most tubules contain large primary spermatocyte nuclei in diakenesis, which obliterate the lumen completely There is also evidence of regression, since degenerating spermatocytes lie loose in some tubules In the 5 months old animal killed in November the primary spermatocytes are not as numerous as in October and the majority of tubules are in a condition of complete quiescence. The spermatogonia lying among the Sertoli nuclei are large cells similar to the winter spermatogonia of the adult (*d*) *Interstitial Tissue*—The interstitial tissue of the testis in animals with cyclic spermatogenesis appears to vary in amount at different stages of the cycle Owing to the difficulty experienced in estimating the amount of interstitial tissue no attempt has been made to determine whether the volume varies in the ferret The tissue has been examined for mitosis, but at no period of the year were any figures seen Many authors have commented on the absence of division in the interstitial cells (see Rasmussen, 1917, for review), but von Winiwarter (1912) maintains that they divide by a special mechanism resulting in multinucleated cells The one or two examples of bi-nucleated cells in the ferret material may be evidence of division

Tandler and Grosz (1911) state that in the mole the islands of interstitial tissue are largest and are therefore most active from May to August, when the spermatogenic tubules are quiescent, whereas in March when spermatogenesis is most active the interstitial tissue islands are smallest and least active Other authors, however, maintain that the cycle in the interstitial tissue is closely parallel with that in the seminiferous tubules Hansemann (1896) states that the interstitial cells in the woodchuck are hardly represented during hibernation, in spring the tissue is highly developed Ganfini (1903) found that the interstitial tissue in the woodchuck was apparently reduced during hibernation, but points out that this is probably due to reduction in cell size Marshall (1911) noted a great increase in the amount of interstitial tissue in the hedgehog during the breeding season Courrier (1923) also found that the maximum development of interstitial tissue in this animal synchronised with the breeding season In the woodchuck Rasmussen (1917) records development of the interstitial tissue after hibernation, during which it is scarcely discernible He ascribes the increased size of the testis of the rutting animal to the development of this tissue, since the diameter of the seminiferous tubules varies little throughout the year Courrier (1923) found an increase of interstitial tissue in the breeding season in the marmot Regaud (1904) confirmed the observations of Tandler and Grosz on the mole, but Lecaillon (1909) records degeneration of the interstitial tissue in July in this animal The conclusions are more conflicting since some workers consider apparent changes in the amount of the interstitial tissue, while others deal with signs of glandular activity

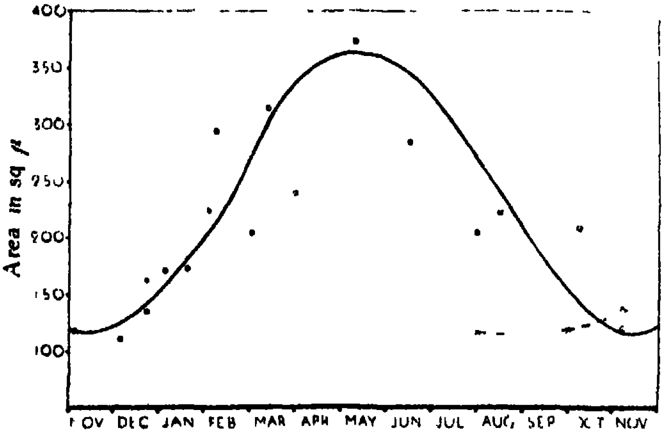
To determine whether cyclic secretory activity occurs in the interstitial cells of the ferret the following methods have been used —

(i) Measurement of the area of individual interstitial cells at different periods

(ii) Comparison of the nuclear-cytoplasmic ratio.

(iii) Comparison of the fat content of the cells

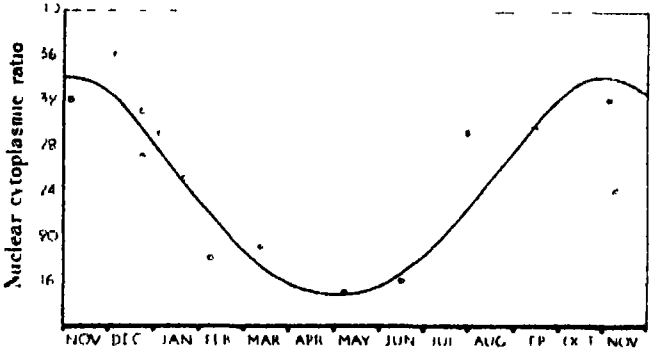
Variation in Size of the Interstitial Cells —The average area of the interstitial cells was obtained for each animal from camera lucida drawings of 25 cells In November and December the area varies between 110 and 120 sq μ, in May they have increased to 370 sq μ The intermediate stages fall fairly regularly on a curve rising steeply during January, February, March and April In the middle of June the area has fallen to 285 sq. μ, and the cells continue to decrease in size until November



TEXT-FIG. 3.—Seasonal variation in area of interstitial cells

Both the nucleus and cytoplasm take part in this cyclic variation The nucleus in November has an average area of 29 sq μ, while in March it measures 52 sq μ (Table I) The increased area of the cells in the breeding season is, however, mainly due to increase of the cytoplasm. Text-fig 4, in which the nuclear-cytoplasmic ratio is plotted throughout the year, shows the extent to which the cytoplasm increases during the breeding season, it also gives some indication of the state of activity of the cells, since the increase in the cytoplasm is due partly to the accumulation of the products of secretion and possibly to an enlarging of the sphere Benoit (1926) has shown that, in the cells of the epididymis of mouse and guinea-pig, the nuclear-cytoplasmic ratio decreases during the prepubertal period until it becomes a constant relation correlated with the continuous glandular activity of the cells in the adult. Following castration, the glandular activity of the epididymis ceases and the nuclear-cytoplasmic ratio rises to that characteristic of the prepubertal animal

The nuclear-cytoplasmic ratio in the interstitial cells of the ferret would thus indicate that in November and December there is very little secretory activity, in January, February, and March the cells are actively secreting After this period, accumulation of fat keeps the area of the cytoplasm large, and cell size alone cannot be used as an index of secretory activity The nuclear volume is already declining in April and the time of most intensive secretion is probably between January and March



Text-fig 4—Nuclear cytoplasmic ratio of interstitial cells

Fat in the Interstitial Cells—In Flemming-fixed testis the cytoplasm of the interstitial cells contain droplets of osmicated fat In Bouin-fixed material their presence is indicated by the vacuoles in the cytoplasm Fat is present in the interstitial cells of many animals, but is absent in the pig and the opossum (Whitehead, 1908) Its function has been variously interpreted Earlier workers suggested that it was passed into the Sertoli cells to be used as food by the developing germ-cells, but most later workers have discarded this view Ganfini (1902) and Loisel (1902) consider that the fat is used to form the internal secretion of the interstitial cells Other cells of internal secretion possess droplets of fat which blacken with osmic acid, for example, the cells of the corpus luteum and of the adrenal cortex, and the rôle of these fats in the formation of secretion has been discussed by Goormaghtigh (1927)

Rasmussen (1917) found that fat was always present in the interstitial cells in the woodchuck, but increased enormously during the breeding season Courrier (1923) describes the same variation in the amount of fat in the interstitial cells of the hedgehog, marmot, and bat, but in the mole the cells possess a large amount of fat at all seasons of the year In view of the conclusions of Long and Evans (1922) and Deanesly (1930), it has been assumed in this study that the nature of the fat droplets is some indication of the state of activity of the cells

The present ferret material does not include Flemming-fixed testes for October and November In December fine granules of blackened fat occur in the interstitial cells During January these have increased in number and are distributed throughout the cytoplasm, fig 11, Plate 12 A few cells have coarser droplets, but the fine granulation is more general The granulation remains fine, but becomes increasingly profuse during February, March and April In May many cells possess blackened masses arising probably from the fusion of smaller droplets This condition becomes more general during the summer, fig 12, Plate 12 By the middle of August a large proportion of the cells have coarse masses of fat completely filling the cytoplasm, but other cells in early stages of fat accumulation have finer globules interspersed between the denser masses If the accumulation of coarse irregular masses of fat is a sign of decreasing activity of the cells, the activity of the interstitial cells of the ferret falls off once the breeding season has begun

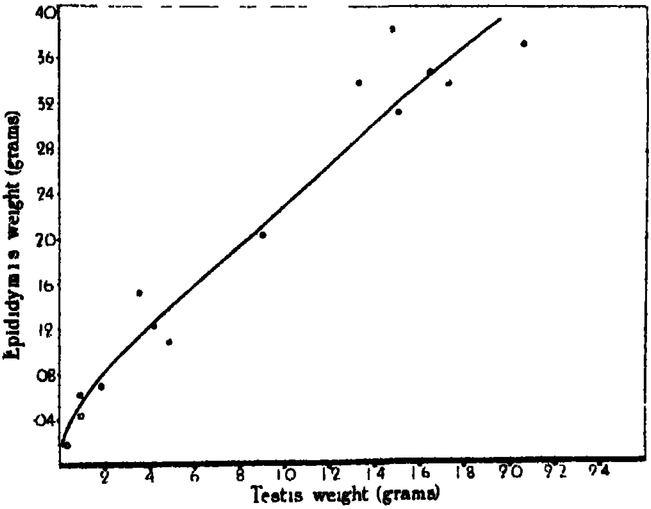
Pigment in the Interstitial Cells —Pigment occurs in the interstitial cells of the testis of a number of mammals, and in animals with seasonal variation in the fat in these cells, the amount of pigment varies inversely with the amount of fat Pigment was found in the interstitial cells of only one of the ferrets—F 23, killed in April In this animal the interstitial cells possessed irregular masses which appeared yellow and refractive in Bouin-fixed material It has been claimed by Courrier (1923) in the testis and by Mulon (1913) in the corpus luteum, that the fat is converted into pigment during the involution of the cells Rasmussen (1918), however, considers that the pigment arises from fuchsinophil granules

Interstitial Tissue in Immature Animals —The interstitial cells grow gradually in the immature animal and the cells in the 5 months old ferret killed in November are rather larger than in the adult of the same month The nuclear-cytoplasmic ratio decreases rapidly between 2–5 months old, and, since the nucleus scarcely varies in size, this is largely due to increase in the amount of cytoplasm

In the 2 months old animal, fat is scarcely discernible ; there are fine granules, brown or grey, but not black At 4 months old the cells show sparse fine granules of blackened fat, and in November these are more plentiful It appears, from comparison with December adults, that more fat is then present in immature animals than in the adults Fat occurs in the seminiferous tubules in October, but not in August

IV. *Changes in the Epididymis*

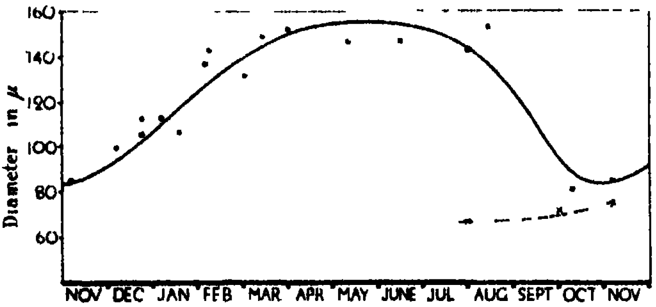
(*a*) *Variation in Size* —Table I gives the weight of the epididymis throughout the year As with the testis, the lowest weight is recorded in November when it is 0 007 per cent of the body weight, while from March to August it varies between 0 035 and 0 039 per cent In text-fig 1 the percentage weight is plotted for the various months The curve rises until the beginning of the breeding season and remains approximately constant throughout the summer months, while the testis weight begins to fall after the first 4 weeks of the breeding season When the epididymis weight is plotted against the testis weight a close correlation is found, text-fig 5, during the period of growth, the variation at the higher weights can be accounted for by the difference in the behaviour of the two organs during the breeding season



Text fig 5 —Testis and epididymis weights

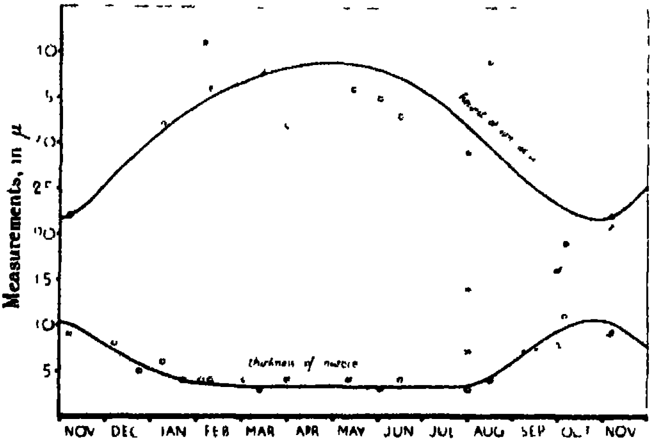
(*b*) *Diameter of the Epididymis Tube* —The cyclic variation in the size of the epididymis is connected closely with the histological appearance The diameter of the tube increases with the onset of activity in the testis and decreases again at the end of the season Fig 3, Plate 10, shows a section of the body of the epididymis in the resting condition; the lumen is small, the circular muscle layers form a wide band, and the fibrous tissue is greatly developed In the breeding season the diameter of the tube increases greatly, the circular muscle layers become thin and the fibrous tissue scarcely appears between the coils of the tube, fig 4, Plate 10 These changes take place in all the segments of the tube, but for purposes of comparison measurements were made in the body region in which less individual variation in the coils was found and in which all the cells are secretory   In this region the tube varies from 80 μ in November to 150 μ in the breeding season   In text-fig 6 the figures are plotted against the month of the year

The height of the epithelium increases from 20 μ in the resting condition to 35–40 μ in the breeding season   The width of the muscular layers surrounding the tube varies inversely with the diameter of the tube, being four or five layers deep in the winter months (8–9 μ in width) and falling to two or three layers (3–4 μ in width) in the breeding season, text-fig 7   This change can be explained on the grounds that the fibres merely stretch and contract during the cycle without actually undergoing development   The changes in the epithelial cells indicate, however, that a secretory cycle occurs in the epididymis and that the enlargement of the tube in the breeding season is not due solely to distention by masses of sperms   Other evidence of a secretory cycle is provided by the development and regression of the non-vibratile cilia of the epithelial cells   According to Benoit (1926) and other authors, these threads are intimately connected with active secretion by the epithelial cells, during which droplets pass down them to the lumen   In the quiescent state these threads are difficult to distinguish, lying crumpled and matted on the surface of the cells   They measure approximately 1 7 μ in November when the height of the epithelial cell is 22 μ, while in the breeding season, when the epithelial cells are 35–40 μ in height, the threads measure as much as 20–30 μ, figs 9 and 10, Plate 12



Text-fig 6 —Variation in diameter of epididymis tube

(c) *Epididymis in Immature Animals*—As with the testis, the percentage weight of the epididymis falls between the ages of 2 months and 5 months, being 0·013 at 2 months old, and 0·008 at 5 months (text-fig. 1)   Nevertheless, there is gradual growth in the epididymal tube and its epithelium, so that in November a close approximation is reached between the immature and the adult animal The muscle layers during this period increase slightly in thickness from 7 μ to 9 μ



Text fig 7—Seasonal variation in height of epithelium and thickness of muscle layers of epididymus

V *Changes in the Vas Deferens*

The diameter of the vas deferens in the ferret varies slightly throughout its length, but there is no marked dilation into an ampulla The lining epithelium bears non-vibratile threads except in the part of the tube immediately above the urethra These threads are about 6 6 μ long in the breeding season and are matted with secretion from the epithelial cells, in anœstrus the threads almost disappear. The muscular wall of the tube consists of an outer band of longitudinal muscles, and an inner, narrower band of circular fibres There is no inner layer of longitudinal muscle fibres

The vas deferens is a region of intense secretory activity (Benoit) and has been shown to be directly affected by the hormone of the testis Thus regression of the vas deferens after castration has been noted by several authors, and Vatua (1930) has shown that the degenerative changes can be prevented by the injection of testis extracts No observations, however, have been made on the changes in this organ in animals with seasonal reproductive activity The vas deferens in the ferret shows cyclic regression and development correlated with the changes in the testis During the winter months the diameter of the tube, including the muscular layers, is 240–280 μ, while in the breeding season it increases to 420–480 μ This change is due to enlargement of the lumen, to increase in the height of the epithelium, and to development of the muscular layers, figs 13 and 14, Plate 12

The vas deferens material is unfortunately incomplete, none being available for the anœstrus before December Probably, however, the increase in diameter of the tube and in the width of the muscle layers is extremely rapid, the diameter is 240 μ at the end of December and has reached 450 μ at the beginning of February (see Table I) It is intended to make a more detailed study of the seasonal changes in the vas deferens in other mammals to determine when growth commences in preparation for the reproductive season

VI *Changes in the Penis*

An attempt has been made to determine whether there is a cyclic variation in the weight of the penis since it seems probable that the erectile tissue would hypertrophy during the breeding season In the ferret, however, where an *os penis* is present, size regression during anœstrus could only be partial The weight of the penis was obtained from 11 animals killed between December and June, for one of these animals, however, the body weight is not available The weight, calculated as a percentage of the body weight, varies from 0 082 per cent to 0 314 per cent (see Table) These observations are inadequate to suggest that the penis increases in size during the breeding season, but more data might show a cycle in the weight of this organ

VII *Discussion*

The observations recorded for the male ferret show that the changes in the testis and accessory sexual organs consist of gradual growth and preparation for the breeding season, after which the reproductive system returns to the anœstrous state There is a shorter period of quiescence than in the female and a more extended reproductive season, since males are producing spermatozoa, which presumably would be capable of fertilising the female, at least 6 weeks before the female is ready for breeding Possibly there is no period in the cycle of the adult male when activity ceases completely in the seminiferous tubules, the production of spermatocytes seems to continue, although at a slower rate, during the least active period in October and November Spermatozoa, however, are absent for 6 months in the year In the epididymis, growth for the next breeding season commences soon after regression from the last is completed, but there is a prolonged period of glandular inactivity.

Table I

Number of ferret.	Date	Body weight.	Testis						Epididymis					Vas deferens.	Penis.	
			Weight	Per cent. body weight.	Mean diameter of tubules	Interstitial cells			Weight	Per cent body weight	Mean diameter of tube	Height of epithelium	Thickness of muscle	Diameter	Weight.	Per cent. body weight
						Area.	N/C ratio	Area of nucleus								
		gm.	gm.		μ.	sq μ		sq μ	gm.		μ.	μ.	μ	μ	gm	
F.17	Nov 5	1100	0 178	0 016	77	118 2	0 32	28 8	0 071	0 007	84 9	22	9	—	—	—
F 18	Dec. 5	1200	0 355	0 030	105	110 9	0 36	29 7	0 146	0 012	99 6	30	8	—	0 966	0 082
F 19	Dec 24	1000	0 424	0 042	129	162 1	0 27	34 4	0 122	0 012	105 5	28	5	241	1 064	0 108
F 20	Dec. 24	800	0 498	0 062	146	135 4	0 31	31 8	0 108	0 014	112 8	—	—	—	1 466	0 183
F.21	Jan 6	1200	0 901	0 075	145	171 5	0 29	38 7	0 217	0 018	112 0	32	6	276	1 772	0 148
F 10	Jan. 20	675	0 641	0 095	126	172 4	0 25	34 8	—	—	106 6	30	4	—	1 320	0 196
F.22	Feb. 4	1400	1 742	0 125	165	223 6	0 29	50 7	0 335	0 025	137 5	41	4	452	2 100	0 150
F 8 Lt.	Feb. 8	—	2 140	—	179	294 5	0 18	44 3	—	—	143 1	36	4	—	—	—
F 23	Mar 2	900	1 530	0 170	147	203 8	0 23	43 4	0 312	0 035	131 6	31	4	422	2 760	0 307
F 12	Mar 12	—	1 380	—	194	314 3	0 19	52 0	—	—	149 2	38	3	434	—	—
F 24	Apl 1	940	2 062	0 219	181	240 8	0 17	35 0	0 368	0 039	152 4	32	4	—	2 950	0 314
F.25	May 9	1000	1 320	0 132	143	371 5	0 15	48 1	0 337	0 034	146 9	36	4	428	1 810	0 181
F 8 Rt.	June 2	—	1 480	—	157	—	—	—	—	—	143 1	35	3	—	2 370	—
F.26	June 16	940	1 640	0 174	156	285 9	0 16	39 9	0 343	0 036	147 8	33	4	470	2 350	0 250
F 13	Aug. 1	1100	1 469	0 133	175	205 1	0 29	47 3	0 384	0 035	142 8	29	3	—	—	—
F 3	Aug 14	—	—	—	181	222 3	0 22	40 3	—	—	152 2	33	4	—	—	—
F 1	Oct. 7	—	—	—	91	208 9	0 26	34 0	—	—	80 9	19	11	—	—	—
F 72	Aug. 1	150	0 034	0 023	58	116 1	0 29	26 2	0 019	0 013	67 1	14	7	—	—	—
F 15	Oct. 1	—	0 092	—	63	118 3	0 30	27 5	0 064	—	71 7	16	8	—	—	—
F 16	Nov 5	560	0 087	0 016	76	138 5	0 24	26 7	0 043	0 008	75 3	21	9	—	—	—

Although it is assumed that the anterior pituitary body plays a similar part in the regulation of the male reproductive organs to that which it plays in the female, the administration of the gonad-stimulating substances has so far failed to produce spermatogenesis either in the immature animal or in the anoestrous male Bissonnette (1931) however, has succeeded in inducing spermatogenesis in starlings by exposing them to increased light periods and has obtained fully active testes in December when control birds are quiescent

Most authors consider (see Lipschütz, 1924, and Marshall, 1922, for bibliographies) that the interstitial cells produce a secretion controlling the development of the accessory and secondary sexual organs Rasmussen, however, in a recent review (1928) throws doubt on the validity of this conclusion Whatever the function of the secretion produced, the morphological changes observed in the interstitial cells in animals with seasonal reproductive activity are undoubtedly connected with a cyclic production of secretion In the ferret, the cycle in the interstitial cells and accessory organs follows very closely the cycle in the seminiferous tubules Courrier (1923, *a*), however, found that the interstitial cells in the bat appeared to reach their maximum activity after spermatogenesis had begun This he correlates with the cycle in the epididymis where secretory activity reaches its maximum after the end of the breeding season Craig-Bennett's observations on the stickleback (1931) are of interest in this connection He was able by variation of the temperature to dissociate the spermatogenic cycle from that in the accessory organs, and found that development of the sexual characters was always accompanied by heightened activity of the interstitial cells He concludes, however, that the secretion of the interstitial tissue alone is not sufficient to cause development of the accessory sexual characters and that its activity is not conditioned by a threshold temperature The rise of temperature does not directly affect the interstitial tissue but acts through some other glandular organ, possibly the pituitary or thyroid bodies

VIII *Summary*

(1) Observations have been made on the state of the testis, epididymis, vas deferens, and penis in 16 adult and 3 immature male ferrets, killed at different periods in the year

(2) The testis and accessory sexual organs are in full activity from March to July During August, September and October regression takes place After a period of quiescence, preparation for the next breeding season begins

in December and results in gradual growth in testis, epididymis, vas deferens, and probably also in the penis

(3) The weight of the testis and epididymis, calculated as a percentage of the body weight, is high during the breeding season and falls to the lowest point in November, text-fig 1 The increase of weight in the epididymis is closely correlated with that of the testis, text-fig 5

(4) The diameters of the seminiferous and epididymal tubules show similar cyclic variation during the year text-figs, 2 and 4

Connected with the increase in the size of the epididymal tubules in the breeding season is an increase in the height of the epithelium and a reduction in thickness of the muscle layers, text-fig 7

(5) The vas deferens has a greater diameter in the breeding season than in the winter months, due largely to growth of the muscular coats

(6) The interstitial cells of the testis undergo cyclic changes throughout the year. They are large and filled with fat in the breeding season, and small, with little fat, during anœstrus, text-fig 3

I wish to express my gratitude to Dr A S Parkes for supplying the material, for his generous help and guidance during the course of this research, and for reading the paper My thanks are also due to King's College, London, for a grant defraying the histological expenses of the work

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DESCRIPTION OF PLATES

Abbreviations

BV, blood vessel, *CM*, circular muscles, *CS*, cytoplasmic syncytium, *DGM*, degenerating masses, *EP*, epithelium, *F*, fat, *IC*, interstitial cells, *LM*, longitudinal muscles, *SN.*, Sertoli nucleus, *SPC*, spermatocyte, *SPG*, spermatogonium, *SPT.*, spermatid, *ST*, seminiferous tubule, *TP*, thread processes

Plate 10

Fig 1—Testis of F 1, killed October 7 Tubules in quiescent condition, interstitial cells small × 80

Fig 2—Testis of F 23, March 2. Tubules in full activity; interstitial cells large × 80

Fig. 3—Epididymis of F.17 in body region, November 5 Quiescent condition × 80

Fig 4—Epididymis of F 24 in body region, April 1 Condition in breeding season × 80

Plate 11

Fig 5—Testis of F 17, November 5, showing "winter spermatogonium" and Sertoli nuclei × 1050

Fig. 6—Testis of F 18, December 5 Spermatocytes becoming numerous. × 1050

Fig 7—Testis of F 22, February 4, showing advanced spermatids × 1050

Fig 8—Testis of F 13, August 1, showing commencement of regression Spermatocytes and spermatids loose in lumen × 350

Plate 12

Fig 9—Epididymis of F 24, body region, showing development of thread processes, April 1 × 1050

Fig 10.—Epididymis of F 17, body region, showing regression of thread processes, November 5 × 1050

Fig 11—Interstitial tissue of F 21. Fat scarce and in fine granules, January 6 Flemming fixation, unstained × 350

Fig 12—Interstitial tissue of F 26 Fat abundant and in coarse droplets, June 16, Flemming fixation, unstained. × 350

Fig. 13—Vas deferens of F 19, showing resting condition, December 24 × 80.

Fig. 14.—*Vas deferens of F.26. Condition in breeding season, June 16* × 80

With the exception of figs 11 and 12, Plate 12, all preparations are of Bouin-fixed material, stained in hæmatoxylin and eosin.

The microphotographs are the work of Mr. D. Kempson.

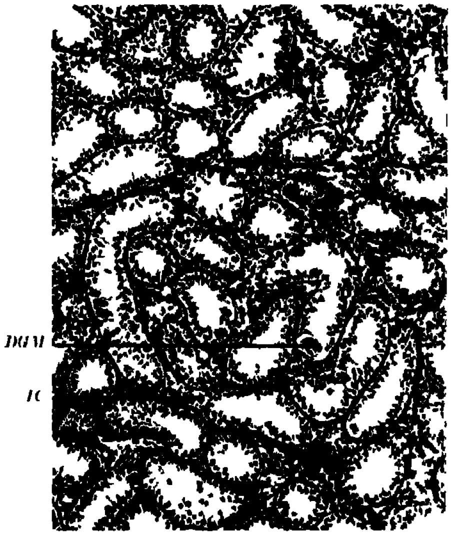
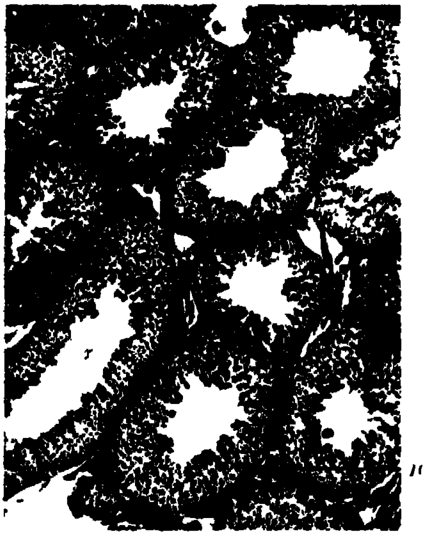


FIG 1



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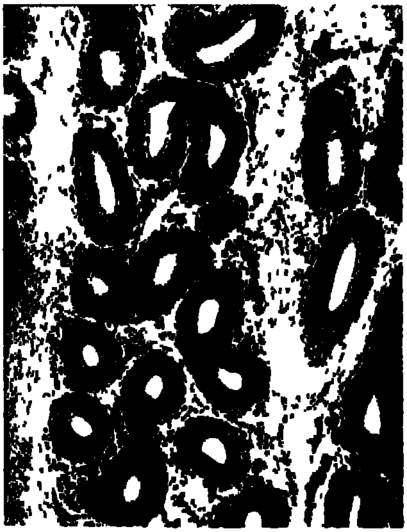

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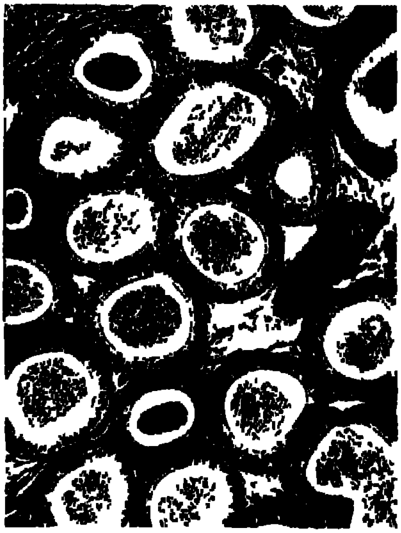

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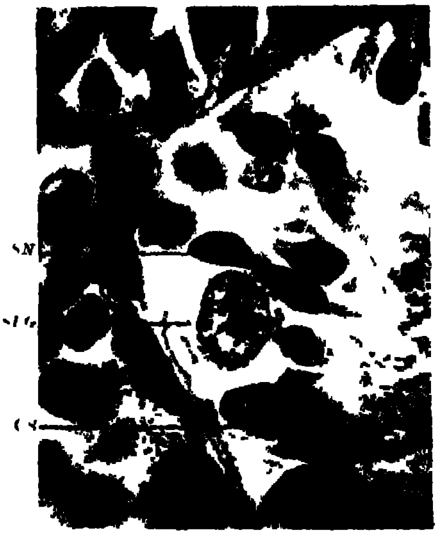

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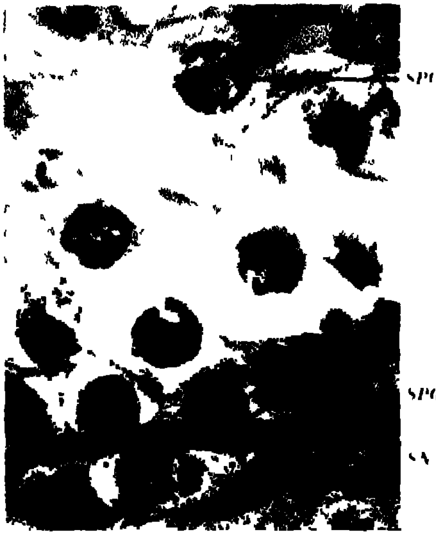

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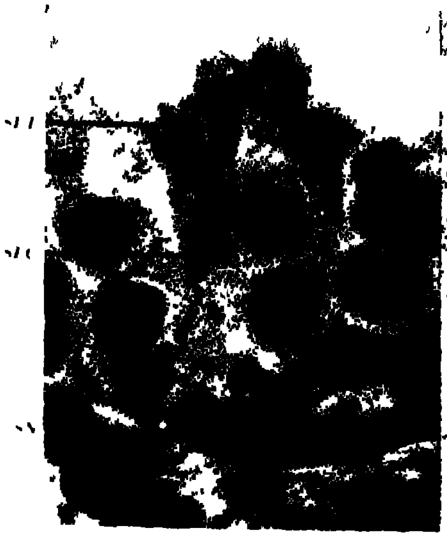

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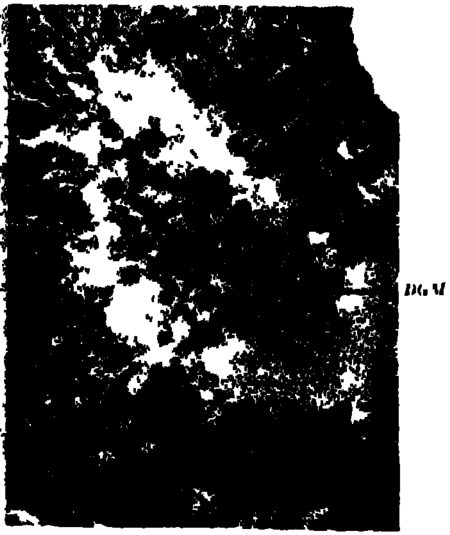

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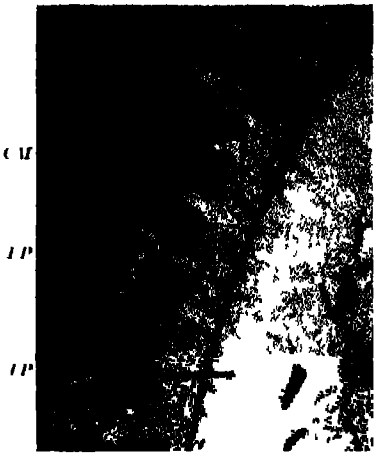

Fig 9

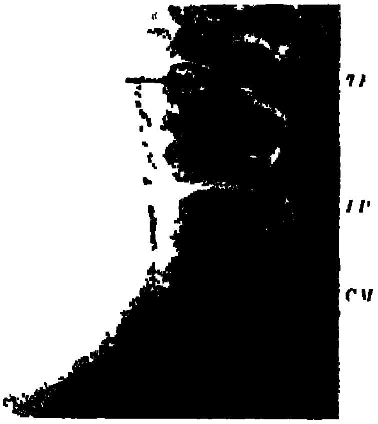

Fig 10

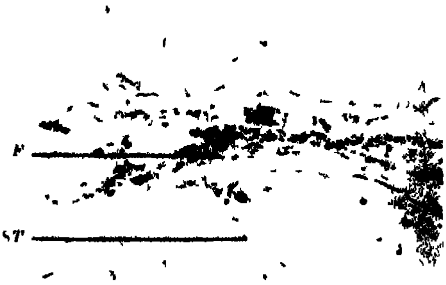

Fig 11

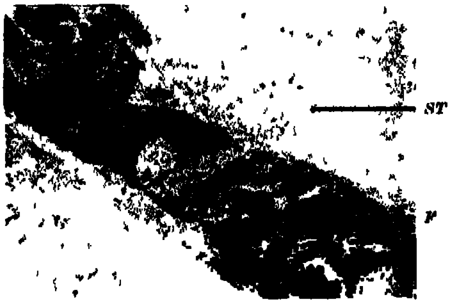

Fig 12

Fig. 13

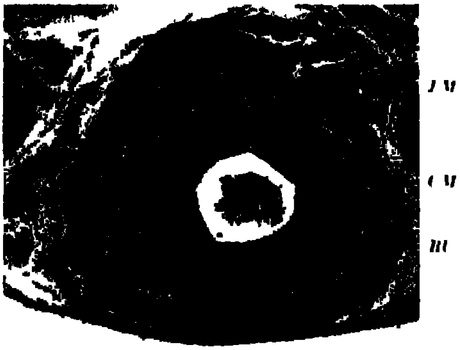

Fig 14

612.61/62:612 014 44

Factors affecting the Breeding of the Field Mouse (Microtus agrestis)
Part I.—*Light.*

By JOHN R BAKER, D.Phil, and R M RANSON.

(From the Department of Zoology and Comparative Anatomy, Oxford)

(Communicated by E S Goodrich, F.R S—Received January 11, 1932)

Introduction

The investigations described in this series of papers were partly experimental and partly observational The first two papers will deal with the experimental work, and the third with the observational The observational work has shown that *Microtus agrestis* has a definite breeding season in the British Isles, extending roughly from March till September This species appeared to be a suitable one on which to experiment for the purpose of finding the factors which control the breeding season, a subject on which so little is known in any mammal Our immediate object has been to find the factors, and not to find how they work There seem to be two sorts of way in which they might work Firstly, one could imagine that it might be physiologically impossible for an animal like *Microtus* to breed below a certain temperature, or in the absence of a certain foodstuff There is, however, a second, quite different way in which such factors might act It is possible that during the course of evolution *Microtus* might have evolved an adaptive response of stopping and starting reproduction when the temperature and food altered with the seasons beyond certain limits One could imagine the natural selection of the offspring of those individuals which responded to the environment by breeding only at the most appropriate time for the rearing of the young In our preliminary investigations we have not attempted to distinguish between these two possible modes of action of factors controlling the breeding season Our object has been to find the factors, irrespective of the way in which they work

We have regarded the problem from the biological rather than the physiological point of view No doubt we could stop *Microtus* from breeding by depriving it of vitamin E, or subjecting it to extremes of temperature; but our experiments were planned in such a way that our animals were never subjected to unnatural conditions They were never fed on unnatural foods,* nor kept at higher or lower temperatures than occur naturally in Great Britain, nor given longer or shorter periods of light, nor more intense light. We were not anxious simply to stop and start the reproduction of *Microtus* at will, but to find what are the natural factors which control its breeding season in a state of nature.

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* With the exception mentioned later in this paper.

The work was done in the Department of Zoology and Comparative Anatomy at Oxford, and we wish to thank Professor E. S. Goodrich, F.R.S., for his interest in it. Dr. F. H. A. Marshall, F.R.S., most kindly visited us during the course of the experiment and gave us his valuable advice. We have always been in close co-operation with Mr. C. S. Elton. Till near the end of the investigation we were financed by a grant from the Empire Marketing Board, allocated to us by the Ministry of Agriculture and Fisheries. We wish to thank the Board and the Ministry for making the work possible. On the expiry of this grant, the University of Oxford made us another grant to enable us to complete the investigation and to keep our stock of *Microtus* alive for further research. We are very grateful for this help. Mr. A. D. Middleton has helped when either of us has been away from Oxford. We could not have obtained the mice without the generous help of the Forestry Commission.

Methods

The stock of *Microtus* was gradually built up during 1930 from specimens trapped on the Forestry Commission areas in Roxburghshire and Merionethshire. The trapping of *Microtus* alive on a large scale is very difficult, because it seems unattracted by any bait, and only enters traps if they are placed in its run. Its lack of interest in bait makes the usual trap-door release unsuitable. We have therefore used the platform-release trap invented by Mr. C. S. Elton. These traps are of zinc with wooden bottoms. Bread must be placed within the traps to keep the mice alive after capture.

During 1930 we learnt how best to breed *Microtus* in captivity. We planned the experiments to start on January 1, 1931, and end on December 31, 1931.

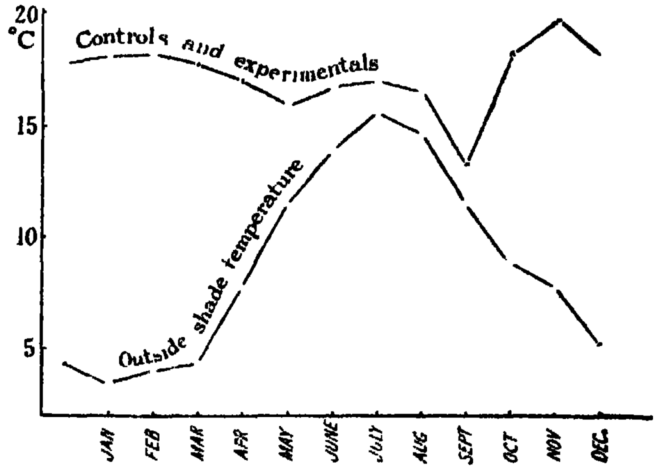
We shall first describe the conditions under which the control mice were kept throughout this period. It was our object to keep the control mice always under summer conditions of temperature, food, and length of day.

The control mice were kept in four boxes, 17 inches long by 16 inches wide by 12 inches high. The boxes were of perforated zinc on creosoted wooden frames, with a metal tray beneath. There was a layer of about ¾ inch of peat moss at the bottom of each cage. Tins were placed in corners for the deposition of fæces. The mice were readily taught to use these by placing fæces in them

A nest box and a revolving wire wheel were provided in each cage The mice made frequent use of the wire wheels, which provided sufficient exercise for the maintenance of good health We wish to thank Mr Vernon Bailey for advice on keeping *Microtus*

The main natural food of *Microtus* during summer is grass We tried to provide the control mice all the year round with growing grass or its equivalent This was easy in summer time For winter use we planted plots of winter-growing grasses, namely, *Lolium perenne*, *Poa annua*, *Poa trivialis*, and *Cynosurus* These species were kindly recommended to us by Professor R G Stapledon In winter time in order to insure that actively growing food was provided cabbage, lettuce or subterranean clover was given in addition to grass The total allowance of food was about 30 grams per day per mouse, most of which was usually eaten The cabbage or other unnatural food did not exceed one-tenth of the total and in summer time none was given A tin of water was provided in each cage

Our object was, of course, to keep the mice at constant summer temperature throughout the year During the summer they were kept in a wooden animal house, and during the cold part of the year in a special hot room heated by hot water pipes It was not possible to prevent the temperature from varying somewhat from month to month The maximum and minimum temperature were taken daily The graph shows the mean temperature for each month The mean temperature for the whole year was 18 7° C (66° F) For comparison, the mean outdoor temperature of each month is also shown in the graph, from data kindly supplied by the Radcliffe Observatory The mean



monthly maximum and minimum temperatures to which our mice were subjected are given in the appendix

Light was provided for 15 hours each day, except Sundays, when it was about 4 hours less (On December 27, 1931, owing to a mistake, the light was on for less than an hour in both the control and the "winter light" conditions, mentioned later) The light was given by one clear glass electric gas-filled bulb of 60 watts, situated about 3 feet above the cages There was sometimes also some diffuse daylight during the period when the electric light was on. The humidity was read daily about 11 a m by means of a hair-hygrometer, which was corrected occasionally by a wet and dry bulb thermometer The mean relative humidity for the whole year was 84 The mean monthly figures are given in the appendix.

It will be observed that we kept the control mice throughout the year on summer food, at summer temperature (or a slightly higher temperature), with summer period of exposure to light

One adult male was kept in each cage with from one to three females Our object was to keep three females in each cage, but we had not a sufficient stock for this purpose Weekly counts showed that the average number of females under control conditions was 7 5, instead of 12

The following precautions were taken to prevent the possibility that our control mice might not be a random sample Every month two females, chosen at random, were removed, and two new ones substituted, which had already spent at least 3 weeks in store cages under control conditions. No mouse was ever introduced into a control cage unless it had already had 3 weeks of acclimatisation to control conditions, separate from mice of the opposite sex It was decided not to change the males so often, as a new male always attacks the females, and deaths and disturbance of reproduction result The males were therefore removed, killed, and examined for fecundity every 3 months Fresh ones, which had already been subjected to the standard acclimatisation, were substituted

When a mouse died or appeared ill or old, it was removed, and a new one, which had been acclimatised, substituted.

Every week all females were examined, and those that were obviously pregnant were removed, and kept in small cages under control conditions until the birth of the young (or the absorption of the embryos) New acclimatised females were substituted

All the control mice which started the experiment on January 1, 1931, had already been acclimatised for the standard 3 weeks or more. On the conclusion of the experiment, on December 31, 1931, the males were killed, and the females kept under control conditions for 20 days to allow any pregnant ones to produce young

We have now described our controls in detail We also kept three sets of experimental mice The first set was exactly similar in every respect to the controls, except that only 9 hours of electric light was allowed daily instead of 15 We call this the winter light experiment Another set was in every respect similar to the controls, except that the animals were subjected throughout to winter temperature Another set differed only from the controls in that winter food was provided instead of growing grass The winter temperature experiment and the winter food experiment will be reported on in the next paper in this series It will be noticed that we have made the conditions of our experimental mice differ from the control conditions in one factor only in each case, either light, or temperature, or food

We must stress the fact that our winter light mice were kept in four cages of the same construction as the control cages, with the same bedding and furniture, and that the temperature, food and humidity were the same The average number of females under winter light conditions was 7 8 The regular removal and replacement of male and female mice was carried out in exactly the same way as with the controls, and the standard 3 weeks of acclimatisation to the winter light conditions was always carried out before the introduction of new mice into the cages The only difference from the controls was that they were subjected to 9 hours of electric light daily instead of 15 The electric light bulb used was exactly similar to the one used for the controls

The two females removed each month from the control cages were always acclimatised to winter food conditions, and then introduced into the winter food experimental cages This circulation of female mice extended throughout our experiments The mice were circulated as follows every month, the usual 3 weeks of acclimatisation being invariably adhered to before the introduction of mice into the experimental cages —

Controls to winter food
Winter food to winter light
Winter light to winter temperature
Winter temperature to controls

The mice removed from the control and experimental cages each month were selected at random The purpose of this constant circulation of mice was to rule out chance as far as possible If we had not circulated the mice, there would always have been a remote chance that the mice under one of the sets of conditions had inherently different breeding capacities from the others Altogether about 65 females passed through the control cages and about 60 females through the winter light cages

Results

The results of the winter light experiment are very surprising We had not anticipated that the difference between 9 and 15 hours of light daily would be sufficient to have a considerable effect on reproduction The full breeding records of the control and winter light mice are shown below There were, on the average, 7 5 control females throughout the year in the control cages These produced in the year 47 young in 14 pregnancies In addition there were 10 pregnancies which ended in the absorption of the embryos in the uterus (or possibly, in some cases, in the young being eaten at birth without leaving any trace) There were, on the average, 7 8 females in the winter light cages These only produced 8 young, in three pregnancies There was also one case of absorption of embryos In the table below, the sign "0" indicates a pregnancy resulting in the birth of no young, owing to absorption The date is the date on which the pregnancy was first observed The numbers indicate the number of young born, the date being the date of birth

Date	Controls	Experimentals	Date	Controls	Experimentals
1931			1931		
January 20	4	—	August 5	0	—
February 18	1	—	August 14	0	—
March 4	0	—	September 18	4	—
March 14	4	—	September 22	3	—
March 18	3	—	October 1	—	2
March 24	4	—	October 6	4	—
April 16	3	—	October 8	3	—
April 27	—	0	October 27	4	—
May 11	0	—	November 5	2	3
May 18	0	—	November 9	0	—
May 18	0	—	November 21	—	3
May 18	0	—	December 7	0	—
July 15	2	—	December 14	6	—
August 5	0	—			

The effect of the lessened amount of light might be an effect on the males only or on the females only The fecundity of the males was determined by dissection It might be thought that it would have been better to test their fecundity by mating them with females under optimum conditions for breeding, but this has been found impossible in practice, for the following reasons

Male *Microtus* will seldom mate at once with strange females in captivity They tend rather to fight them Conceptions do not usually occur until a male has been with strange females for a fortnight or more If he has been under optimum breeding conditions during this fortnight, his fecundity is no evidence of the effect of the experimental conditions upon him

For this reason we have relied upon the weights of the testes and vesiculæ seminales and the abundance and activity of the sperms as criteria of fecundity The organs were weighed in fresh condition The contents of the epididymis were examined in alkaline glucose-saline on a hot stage under the microscope The number of the sperms was recorded as abundant, few, or none The activity of the sperms was recorded according to a scheme in which the maximum activity is 3+, no activity whatever is 0, and 3, 2+, 2, 1+, and 1 are intermediate degrees This scheme of grading is fully explained elsewhere (Baker, 1931)

The results are shown in the table Occasional shortage of acclimatised males prevented us from being able to keep strictly to our plan of killing four

Table showing Reproductive Condition of Males when Killed for Examination

Weight	Weight of testes	Weight of vesiculæ seminales	Abundance of sperms	Activity of sperms	Fecundity
		Controls			
grams	milligrams	milligrams			
43	560	110	Abundant	3	Fecund
33	320	200	"	2	Fecund ?
28	350	160	"	3	Fecund
34	550	90	"	3	Fecund ?
30	270	70	"	3	"
33	420	200	"	3	Fecund
31	450	110	"	3	"
30	250	100	"	3	"
25	100	10	Few	0	Non-fecund
		Experimentals			
37	430	100	Abundant	3	Fecund
32	510	130	"	3	"
30	480	110	"	3	"
37	440	270	"	3+	"
38	400	160	"	3	"
34	380	190	"	3	"
36	300	140	"	3	"
26	30	10	Few	0	Non-fecund
27	50	10	None	—	"
23	190	40	Few	0	"
20	170	20	"	0	"
26	240	20	Abundant	3	Fecund ?

males every 3 months Several of the males were eaten by the females, and their reproductive organs could not be studied For these reasons the table shows 9 control males and 12 experimentals instead of 16 of each

For comparison, it must be mentioned that wild adult males in winter generally have testes of 10 milligrams or less, vesiculæ considerably smaller still, and no sperms at all In summer, wild adult males weighing more than 23 grams are always fecund

The conclusion to be drawn from the table is that both the control and the experimental males are mostly fecund, and the breeding results cannot be attributed to an effect of shortened light-periods on the males There is more infecundity among the experimentals than among the controls, but there is not nearly enough difference to account for the breeding results

We must conclude that the effect of shortened daily light periods in checking reproduction is mainly an effect upon the females The obvious method of studying the females would have been to prepare and examine vaginal smears, but, unfortunately, the slightest disturbance upsets these wild creatures to a surprising degree, and they do not generally breed when handled every day Further, daily handling would have been contrary to our general principle of keeping them as far as possible under the conditions of their natural wild life

On the conclusion of the experiment we could not kill the females for histological examination, because we required them for a breeding stock to start a further series of experiments along the same lines When the next series is finished, the females will be killed, and the ovaries and other parts examined histologically Till then we cannot tell the way in which the daily light period affects the reproductive organs

Discussion

It is said that lights were used in poultry houses in Spain more than a century ago for the purpose of increasing egg-production The subject was first scientifically investigated in the U S A, at West Washington Agricultural College, Seattle, and the State Agricultural College, New Brunswick It was shown that winter egg-production could be increased by artificial illumination Rowan (1927) was the first to show that the length of the daily light-period affected the reproductive organs of male birds His work was on juncos, whose testes were caused to swell during the winter by artifically increasing the daily light-period. His more recent studies (1928) caused him to suppose that light did not have a direct effect, but that increased daily light-periods gave opportunity for more exercise, and that exercise was the controlling factor. These experiments, however, were on a very small scale, only three controls being used They can therefore not be regarded as conclusive Bissonnette (1930) has made similar studies on male starlings on a larger scale His results were the same as far as the effect of increased daily light-periods is concerned , but he found that increased periods of enforced exercise did not stimulate the testes He has also shown (1932) that red light has more effect than green in stimulating the growth of the testes

While our work has been in progress, Dr Bissonnette has been extending his investigations to the ferret He has kindly kept us informed of his results He finds, as we do, that the female is more affected than the male Perhaps it will be found that mammals differ from birds in that the male is less susceptible to the effects of light on reproduction than the female

It seems improbable that light has a direct controlling effect upon the breeding season of all wild mammals The mole and nocturnal mammals are unlikely to be affected In these forms some other factor or factors must control the length of the breeding season It is not impossible, however, that Eskimo women are affected by the long winter darkness

Summary

The shortening of the daily period of exposure to light from 15 hours to 9 hours almost prevents reproduction in the field mouse, *Microtus agrestis* It is the female that is chiefly affected

APPENDIX

Table showing the Temperatures and Humidity to which the Control and Winter Light Mice were exposed

Month	Mean maximum temperature	Mean minimum temperature	Humidity.
December, 1930	19 2	16 2	72
January, 1931	19 9	16 2	72
February, 1931	19 7	16 6	68
March, 1931	19 4	16 1	65
April, 1931	18 3	16 3	83
May, 1931	18 4	13 6	81
June, 1931	20 9	12 5	76
July, 1931	20 4	13 5	78
August, 1931	20 2	13 0	77
September, 1931	16·6	10 1	83
October, 1931	19 8	16 7	82
November, 1931	21 7	18 0	81
December, 1931	20 2	16 1	87

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Modification of Mammalian Sexual Cycles. Reactions of Ferrets (Putorius vulgaris) *of Both Sexes to Electric Light Added after Dark in November and December* *

By THOMAS HUME BISSONNETTE, School of Agriculture and Experimental Zoology Laboratories, University of Cambridge, England

(Communicated by F H A Marshall, F R S —Received January 14, 1932)

[PLATE 13]

Introduction

Studies by Rowan (1930 and preceding papers) and Bissonnette (1931, *a*, *b*, 1932, *a*, *b*, and preceding papers) and Bissonnette and Wadlund (1931) have shown that in juncos, canaries, crows and starlings, at least, among birds, the sexual cycle is conditioned to a great degree by daily light ration Sexual activity in both males and females can be modified at will by altering the daily light ration with controlled electric lighting from ordinary incandescent bulbs Gonad changes, controllable in this way, are closely correlated with migrations in juncos and crows, as demonstrated by Rowan (1930, and preceding papers), who, however, maintains that it is the added periods of muscular exercise, rather than the light, as such, that condition these changes, in spite of the fact that it is always the added light he uses, rather than exercise, to get his effects. Bissonnette disagrees, and holds it is the light that is the major factor in conditioning the changes in sexual cycles

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* Aided by grants from the Committee for Research in Problems of Sex, and Grants-in-Aid Committee of the National Research Council of the United States of America, administered by the author, who wishes to express his thanks.

It has been found, also, that intensity and colour, or wave-length, as well as daily period of light are factors in conditioning sexual activity in starlings, and the method of increase of light also plays a part (Bissonnette 1931, *b*, 1932 , Bissonnette and Wadlund, 1931)

The study to be described here was undertaken to find out whether or not sexual cycles in mammals with long winter periods of sexual quiescence or anœstrum, like those of the starlings, are conditioned in like manner by daily period of light—either daylight or electric light

Ferrets (*Putorius vulgaris*) appeared to be almost ideal animals for such a study, because the sexual condition of females can be learned by casual inspection of the vulva This renders operation or further observation unnecessary to find out if œstrus is being induced In males, size and position of the testes, as determined by palpation, give a relatively rough indication of the sexual condition, and the mating reaction and fertility or non-fertility of matings are the best possible criteria of the conditions of the germinal epithelium and the endocrine gland of the testis The animals are small and easily kept for breeding and experimental purposes on a standard routine system of feeding and care, and they are usually easily handled The complete normal sexual cycles of females have been studied in this laboratory and described in all details by Marshall (1904) and Hammond and Marshall (1930), and the presence of these investigators makes study of this problem here most effective The male cycle has been well described by Allanson (1931)

Normal Cycles in Ferrets

In females, anœstrum lasts from August till March , œstrum, in the absence of the male, from March till August, accompanied by great swelling of the vulva and a secretion of serous fluid at the receptive period This swelling subsides during anœstrum, during pregnancy, or pseudo-pregnancy, which follows ovulation without fertilisation, and lasts as long as real pregnancy. Ovulation occurs only after prolonged coitus, with either fertile or sterile (vasectomised) males. Successful coitus lasts from less than half-an-hour to 3 hours and is preceded, usually, by an hour or more of very sadistic courtship. The swelling of the vulva recurs with a second œstrus, following on pregnancy or pseudo-pregnancy, should it occur early in the breeding season Complete correlations of all accessory sex-organ changes in females with the ovarian changes and condition of the vulva have been determined and described (Marshall, 1904 ; Hammond and Marshall, 1930)

In males, there is a similar, but shorter, quiescent period for the testis, accompanied by a reduced condition of accessory sex-organs, lasting from October till January, when no sperms are produced or found in the epididymis Testes reach their maximum size, in relation to body weight, in March , their minimum, in October Germ-cells have been found to increase in November and December (Allanson, 1931)

This increase in germ-cells in November and December makes it seem somewhat doubtful, to begin with, that the increasing light ration of late winter and spring constitute as great a factor in conditioning the male sexual cycle in the ferret as it has proved to be in starlings (Bissonnette, 1930, *a*, 1931, *a*, *b*, 1932 , Bissonette and Wadlund, 1931) That this is so will appear from what follows

Material and Methods

On October 12, 1931, three male and three female ferrets were placed in cages in two vertical rows, males in one row and females in the other, without nesting boxes, and with shavings for bedding in amounts judged to be too small to permit the animals to bury and hide themselves away from the light In front of the cages, and slightly above the level of the floors of the two top ones, and far enough back from the cages to permit direct electric light to reach the backs of the lowest ones, a 200-watt incandescent electric light bulb, emitting light of approximately 2660 lumens intensity, was fixed in a clamp This one light for all six cages was controlled by a time switch, so regulated that it gave continuous light from 5 to 11 o'clock each night On November 24, this was changed to run from 4 30 to 11, to compensate for the natural shortening of the daily daylight period The approximate light rations in foot candles, given to the front and back of each cage by this light, are given in Table I Daylight was freely admitted to the cages by day through a large window toward which they faced

The room was not artificially heated in any way apart from the lamp, and some of the windows in the animal house were open at all times Since the room was separated from the other rooms of the animal house only by a partition about 10 feet high, with common space above that, temperature in the room rose but little

In a similar room, across the narrow corridor, but lighted by two windows by day, three females and two males were placed as controls, and fed on rations similar and approximately equal to those of the experimental animals. Controls received no light after sundown and had nesting boxes to curl up in, for warmth, which probably reduced the daily light rations somewhat Room temperature was approximately equal to that where the experimental animals were kept After November 24 there was a slight increase in food rations to all animals, later reduced, because it was noted about that time that males undergoing light treatment were losing weight or getting thinner on the common ration, while females gained on it However, no records of weights were kept previous to that date Weighings were carried out at intervals thereafter, to test the relative gains or losses in weight on the rations, which were kept approximately equal by measuring, but not weighing Data concerning the animals used in this experiment and three other females put on light rations on December 7, are included in Table I

Females were numbered from above downward in the cages, A, B, C, and the males 1A, 1B and 2, respectively A large male was transferred from the control cages to the lighted cage in place of male No 1B, on November 24, after No 1B died, and this male was designated Transfer No 1 in the table Three other females were placed in the lighted room in similar vertical relative positions on December 7, and their data and numbers appear under D, E, F, females from above downward Data for them are incomplete

Observations and Results

Macroscopical —On November 16, at 35 days, it was first noticed that the vulva of female C, in the lowest experimental cage, was definitely larger and more congested than those of control females and than it had been previously Nos A and B were not yet affected Male No 2 also in the lower cage, had his testes somewhat larger than before and still lying farther forward in the groin than the scrotum The other males, 1A and 1B, were thinner than this one and had no appreciable testis size increase

On November 18, at 37 days, male No 1B died from causes unknown, and No. 1A appeared to be ill Testes of the dead male were taken for study and fixed in Bouin's fluid They appeared to be slightly larger than those of controls as judged by palpation of controls, but were not in the scrotum.

On November 19, at 38 days, the vulva of female C was enlarged to about 7/8 maximum size as compared with descriptions and figures from Marshall (1904) and Hammond and Marshall (1930), respectively On seeing the animal, both these investigators pronounced the animal " on heat " The vulva of female A was becoming enlarged also That of female B was not. She was found to be burying herself partly in the shavings, of which she appeared to make better use than the others She was not interfered with, nor her shavings

Table I —Data concerning Ferrets undergoing Experimental Light

Serial No of animal.	Dates of—			Light intensity in foot candles		Weights			Mammary glands. Swelled = + Not swelled = 0	
	Start of light	Swelled vulva, or descent of testes	Copulation	Cage, front	Cage, back	Nov 26	Dec 10	Jan 5		
Females									Dec 21	to Jan 5
A	Oct 12	Nov 23, at 42 days	Dec 10, for 45 mins	14 10	6 40	800	860	890	0	+
B	Oct 12	Dec 15, at 64 days	Dec 21, at 1¾ hours	11 488	5 877	900	960	910	0	+
C	Oct. 12	Nov 19, at 38 days	Dec 11, for 7–8 mins	8 188	4 881	690	770	800	0	+
Control 1	Oct 12	—	—	—	—	—	660	740	0	0
,, 48	Oct 12	—	—	—	—	—	530	610	0	0
,, 49	Oct 12	—	—	-	—	—	640	700	0	0
Males.										
1A	Oct 12	—	—	14 10	6 40	Not weighed		-	Died	N
1B	Oct 12	—	—	11 488	5 877	Not weighed		—	Died	N
2	Oct 12	Nov 23, enlarged	Dec 10, 11 21, as above	8 188	4 881	820	840	Killed, Dec 22		
Control 1	Oct 12	—	—	--	—	—	1000	1080	—	
Transfer 1	Nov 24	—	—	11 488	5 877	1220	1270	1420	—	
			Later Animals							
Females, light									Jan 5	
D	Dec. 7	Jan 6, slightly	—	13 331	8 130	—	740	740	0	
E	Dec 7	Jan 6, 7, more	—	11 377	5 008		710	700	0	
F	Dec 7	Jan 6, nil	—	8 056	4 644	—	700	720	0	

reduced till about December 1, as it was thought this habit might prevent her from coming on heat or delay her till long after the others reached full œstrum This proved to be correct However, on the reduction of the quantity of shavings, she failed to hide from light in them, and she, too, came on heat

On November 22, some one tampered with the switch and the light ran all night

On November 24, at 43 days, female A was on full heat, as shown by the swelled vulva, which at this time was at maximum size and tension in female C as well, and remained so (Hammond and Marshall, 1930) Female C was tried with male No 2, the healthy one, and proved receptive, even teasing the male to make him copulate He tried only a few times and then curled up to sleep in spite of her teasing He was rather a small male and seemed to have difficulty in mauling her For fear he might never be able to master the females, the large male (transfer 1) was put into a lighted cage to be stimulated, from November 24

On November 25 at 44 days, male 1A a pigmented one, died Autopsy showed two abscesses, one on top of the head and one in the throat His whole genital tract was removed for study and fixed in Bouin's fluid The gonads were cut in two across the middle with a sharp razor blade after 24 hours' fixation His hypophysis was fixed in Zenker's fluid These testes were larger than those of the first male to die and than those of controls of similar size He was emaciated

On November 26 placed female C (first to come on heat) with the male, transfer 1, which had only two nights of light treatment She was receptive, and he worried her and made several abortive attempts to copulate, before leaving her to curl up to sleep She teased him His behaviour shows that even normal midwinter males will attempt to copulate with females in œstrus So willingness to attempt to copulate is no very good criterion of sexual potency or even of increased libido

Females A and C were tried every 2 or 3 days with the surviving light-treated male, No 2, who showed increasingly effective attempts to copulate The females remained receptive and their vulvas swelled The newly transferred male made more or less determined attempts to copulate with them also, but did not succeed

On December 5, the light failed to go on, as the switch ran down from lack of winding.

On December 10, at 59 days, the male, No 2, copulated with female A for 45 minutes after securing entry. He was unsuccessful with female C Smears from the vulva of neither female showed sperms after copulation. A few flagella from the epididymis, in a rather motile fluid, with vaginal epithelial cells in oestrous conditions were found The completeness of the copulation was confirmed by Drs Marshall and Walton, and assistants At this time the testes of this male bulged out the scrotum over which the hair had become short This made the testes very prominent He was very keen for copulation

On December 11, at 60 days, he copulated for about 8 minutes with female C When tried with female A, he was unable to do so as she seemed to have a sore vulva and prevented entry

On December 12 I killed a polecat type ferret, bought from a dealer, and fixed the genital tract and hypophysis as before Though he was larger than either of the males that died, his testes and whole tract were smaller than theirs

On December 16, 65 days from the beginning of the experiment, and 16 days after reduction of her shavings, female B's vulva reached about 3/4 maximum size and she was receptive to the males.

On this date the vulva of female A, 6 days after copulation, was paler in colour and smaller in size than at copulation

On December 18, vulvas of females A and C were receding and neither female would take the male The testes of the transferred male, No 1, were in the scrotum and the hair on it was thinning out, making the testes more prominent

On December 21, at 70 days from the beginning of the experiment, and 21 from the time of reduction of shavings for female B, the male, No 2, copulated with her for 1 hour and 40 minutes after entry Smears from her vulva after copulation showed motility and flagella, but no motile sperms

Vulvas of the other two females were much reduced in size

On December 22, at 71 days, I killed male No 2, which had copulated with all three females, and took genital tract and hypophysis for study Testes, penis, and epididymides were much larger than any previously examined in this study, and nearly as large as those of a June ferret kindly lent me from the collection of Mr J Hammond (found on study to be in full spermatogenesis) However, smears made from the fluid of the epididymis of male No 2 did not show sperms, though the motile fluid showed flagella or long cilia, probably broken off from the lining epithelium, and resembling sperm tails

On December 23, the vulva of female B, 2 days after copulation, was paler and less tense than at copulation.

On December 27, vulvæ of all three females were quite small and their mammæ were enlarged—especially in the ones that had copulated earlier.

On January 5, the vulvæ of D and E, two of the three females put on light treatment on December 7, were beginning to enlarge Those of the three pseudo-pregnant females, A, B, C, were still diminishing and their mammary glands beginning to swell Their mammæ and the areolæ surrounding them were quite definitely pigmented in contrast to those of controls, still small and not pigmented This pigmentation of the mammary regions of pseudo-pregnant ferrets has not been described before, so far as we can ascertain The fact in this case has been confirmed on examination by Mr J Hammond and assistants

At the time of writing, the period of gestation, or of pseudo-pregnancy, of females A, B, C, is not complete, so it is impossible to be sure whether we have real or pseudo-pregnancy, though the microscopical condition of the testes of the male used in copulation, and stimulated to such behaviour by light treatment, seems to preclude the possibility of true pregnancy *

Microscopical Findings —No study has been made of the ovaries or other reproductive organs of the females used, because it was deemed better to let them complete the period of gestation or pseudo-pregnancy This time will expire for female A on January 21, and for female B on February 1, approximately

Microscopical studies have been made of testes of two ferrets lent us by Mr John Hammond, of the School of Agriculture, one a June ferret in complete spermatogenesis, one an October ferret, vasectomised the preceding March, and having copulated six times before being killed in October, of a normal male killed on December 12, of a light-stimulated male killed on December 22, after copulating successfully with three females between December 10 and 21, and of two light-treated males that died at 37 and 44 days of treatment on November 18 and 25, respectively While these last two ferrets were not normal, their illnesses were in no way likely to increase the effects of light treatments, but rather to diminish them So they are included in this study because the numbers used were so small that no pertinent evidence should be neglected Limitation of this material must be kept in mind

Without going into details, the findings may be stated as follows Testicular tubules of the June ferret, fig 1, Plate 13, show all stages of spermatogenesis up to and including mature sperms Interstitial cells are moderately large and numerous Since their condition and that of the epididymis has been well described (Allanson, 1931), they will not be dealt with here

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* On completion of the gestation period, no young were born, so that copulation resulted in pseudo-pregnancy only

Testis tubules of the October ferret, fig 2, Plate 13, have large vacuoles in the intra-tubular cytoplasm, and germ-cells are reduced to spermatogonia only Sertoli cells are numerous Interstitial cells are relatively few in number and small in size, as judged by the areas of interstitial cells in section between tubules, as described by Allanson (1931) The lining of the epididymis consists of closely packed short epithelial cells without signs of long flagella or cilia The tube is coiled in close connective tissue with apparently some involuntary muscle tissue close outside the epithelium fig 4, Plate 13

Testis tubules of the December 12 normal ferret, fig 3, Plate 13, are about half as large in diameter as those of the June ferret and about 2/3 as large in diameter as those of the December 22 light-treated ferret killed after copulation Closely packed small interstitial cells occupy smaller areas in sections than in the light-treated ferret, in spite of the much smaller size of the tubules As compared with the June ferret, they occupy larger spaces in comparison with the size of the tubules Spermatogonia and primary spermatocytes are present, but they do not by any means fill the tubules, and many tubule sections do not show primary spermatocytes at all Some tubules still have large vacuoles in their centre like those of the October ferret However, the cytoplasm in the tubules does not show the stringy appearance nor Sertoli cell outlines, so conspicuous in the October ferret The germinal epithelium seems to be just beginning to recover from regression and necrosis occurring in September and October Some parts of tubules have not yet recovered completely, while some have begun germ-cell growth changes The epididymis lining is composed of short columnar epithelium without cilia or flagella Connective tissue and muscle cells are thick, fig 4

Testis tubules of the ferret, 1B, that died on November 18, after 37 days of treatment, fig 5, Plate 13, are almost filled with germ-cells and Sertoli cells Spermatogonia, spermatocytes, and intermediate cells in synizesis, are very numerous Sertoli cell outlines and those of germ-cells are conspicuous. Mitoses are more numerous than in the preceding animal of December 12. Interstitial cells are small, and crowded in relatively small regions between the apparently expanding tubules The epithelial epididymis lining is much taller than in the preceding animal and active multiplication of cells and elongation of the columnar epithelial cells are very marked, fig 6, Plate 13 Flagella or long cilia are present, though not so long as in the December 22, light-stimulated animal, after copulations, fig 9, Plate 13

Testis tubules of the 1A ferret, fig 7, Plate 13, that died on November 25, after 44 days' treatment, have fewer spermatocytes, mitoses, and synizesis stages of germ-cells than the preceding animal. Some tubule sections have great central vacuoles, others a much denser cytoplasm Tubules are smaller, and closely packed small interstitial cells, with little cytoplasm, occupy relatively larger regions between them The epididymis is like that of the preceding ferret, fig. 8, Plate 13

It should be noted in passing that, as a result of his position when curled up to sleep, this animal did not receive as much light on his body as the preceding ferret He was also a pigmented, or "pole-cat" type of ferret, whereas the other was not What the effect of this pigmentation may be on reactions to light is not yet known A similar difference in rate of reaction to the light treatment was found among the females In curling up to sleep, ferrets form a circle with the head and feet inside the ring formed by the body As these ferrets were in cages in vertical rows, the areas of the ellipses formed and of the body exposed to the light were greatest for ferrets in the lowest cages and least for the top ones However, their distances, in the lowest cages, were greatest Since the resultant light dose is inversely as the square of the distance and directly as the intensity of the light source and the area exposed to it, if the skin is the receptor, the two may approximately balance each other If the eyes are the receptors concerned, the position of the head inside the ring and the permeability of the tissues intervening between the retinas and the light would also become factors, regulating the effective dose In any case, this ferret was in the upper cage, in which the area exposed is smallest and the eyes most shielded from the light during sleep, but the distance from the light is least If the skin is the receptor and other factors are kept constant, the effective dose of light is conditioned by the formula, $S=I \times A/D^2$, when S = effective dose, I - intensity of light source, A = area of the surface exposed to light, and D is the distance from the light source to the receptor It is not yet known whether skin of the body, or eyes, or both, act as light receptors in this reaction It is significant, however, that in both sexes the animals in the lowest cages were found to react most quickly to the light stimulus, all other conditions being kept as nearly equal as possible

The testes of the male, No 2, killed on December 22, figs 9 A, B, after 71 days' treatment, and three successful copulations, are larger than any of those described here except the June ferret Testis tubules are about 3/4 as great in diameter as those of the June ferret, fig 1, and 1½ times as great as those of the December 12 ferret, fig 3 Interstitial cells are greatly increased in size and numbers, in the angles between tubules, and their nuclei stain very intensely with iron-hematoxylin after Bouin's fluid Germ-cells are much more numerous than in the normal December 12 ferret and about equal to those of the 1B ferret, that died after 37 days' treatment, fig 5 However, there is much more cytoplasm within the tubules than in the latter, and in some tubules this is very stringy in appearance in stained sections Free cavities are found in most tubules, but some are filled with germ-cells and cytoplasm Both primary and secondary spermatocytes and a few spermatids are present in some tubules No sperms are present so far as noted Both germ-cells and interstitial cells have been stimulated, the latter relatively much more than the former

The epididymis has been greatly stimulated by the treatment, fig 10, Plate 13 The epithelial lining consists of very tall columnar cells with long cilia or flagella The connective tissue and muscle cells, outside the lining, are stretched and thinned out in comparison with other animals studied, from autumn and winter The whole epididymis is enlarged and resembles that of the actively breeding ferret It appears to react on parallel lines with the interstitial cells, though increased germ-cells are also correlated with it

In general, it may be said that the light treatment induces complete œstrus in female ferrets, leading to copulations and at least pseudo-pregnancy accompanied by mammary gland stimulation and areolar pigmentation. The macroscopic changes in the vulva are similar to those of normally occurring œstrus, and so is behaviour In males, treatment results in great stimulation of the interstitial cells of the testis, paralleled by increased size of the penis and epididymis, and particularly of the columnar epithelium lining the epididymis Spermatogenesis is stimulated, but only partly, as compared with the normal cycle Spermatocyte, and even rare spermatid formation, was induced in 71 days, but no sperms Libido was raised to the normal breeding pitch and successful copulations occurred, lasting as long as 1 hour and 40 minutes, which, however, appear to lead only to pseudo-pregnancies Copulations occurred as early as 59 days after the treatments began, though the females were receptive as early as 38 days from the beginning of treatment Partial protection from the light, as afforded by full beds of shavings, reduced the effectiveness of treatment and delayed the onset of œstrus

Discussion

The results described above show that in some mammals, at least, sexual cycles are more or less conditioned by the daily light ration from the visible region of the spectrum This was also shown to be true of birds (starlings, crows, canaries, and juncos) studied by Bissonnette (1931, *a*, *b*, 1932), Bissonnette and Wadlund (1931), and Rowan (1929, 1930, and previous papers) However, in ferrets the females are most completely dependent on light ration for their sexual cycles, while the males respond more slowly or less completely, and require additional treatment of some sort In males there is something wanting or maladjusted, in the treatment given, which is necessary for complete spermatogenesis Accessory sex organs of males respond apparently completely, and mating takes place , but incompleteness of spermatogenic response renders these matings sterile

In birds, on the other hand, the males apparently respond to similar light treatment somewhat more quickly and just as completely as the females No mating or egg-laying as a result of artificial light stimulation out of season has yet been reported, and the deficiency appears to be on the female side in birds However, the ovaries of birds artificially stimulated by light develop large follicles, and their oviducts become similar in size and gross appearance to those of normally breeding females (Bissonnette, and Rowan, as above cited)

Studies by Baker and Ranson (who appear to be the first to study the relation of light rations to sexual cycles in mammals) on voles, show that the females of that species respond to modification of light ration most completely and fail to reproduce on a reduced light ration, while males remain potent and fertile They find that other factors beside light are concerned with sexual cycles in voles, particularly in males (Baker and Ranson, 1932)

Parallel results in experiments with anterior lobe hypophysis implants, extract injections, and replacement experiments, and with prolan (Zondek-Aschheim), suggest that the light-induced sexual cycle reactions, in both birds and mammals, are bound up, at least in part, with anterior lobe hypophysis activity and hormone balance, in both mature and immature animals (Bourg, 1930 ; Brouha and Simonnet, 1930 , Desclin and Brouha, 1931 , Engle, 1931, and preceding papers , Fourcin, 1931 , Hill, 1931 , Kraus, 1930 , Moore, 1930 , Moore and Price, 1931 , Noether, 1930 , Riddle and Flemion, 1928 , Riddle and Polhemus, 1931 , Schockaert, 1931 , Tallent, 1931 , and many others cited by them)

In particular, the results obtained by Hill and Parkes (1930) on the anoestrous ferret, and on the rat rendered anoestrous by dietary deficiencies, by Merriam and Parkes (1929), come close to the reactions here described for ferrets in the anoestrous period, treated with increased light rations, and suggest a fundamentally similar cause

It is impossible to go into all the vast literature on gonad-hypophysis inter-actions here. The studies above cited appear to touch our work most nearly, and their bibliographies will cover most of the pertinent studies.

As suggested to me by Mr John Hammond of this laboratory, it is interesting, even if not pertinent to this situation, that, in birds, the male, and, in mammals, the female, are homozygous for sex and homogametic, and these are the sexes in these classes that are most susceptible to light ration changes and changes in anterior lobe hypophysis, hormone balance or concentration, while the heterozygous heterogametic sexes are less susceptible This may, or may not, have any casual relation to the situation, but, if it has, a genetic basis in the sex chromosomes underlies the difference in reaction of the two sexes to these endocrine and environmental variables

Summary

(1) Between October 12 and January 5, three female ferrets, subjected to 6–6½ hours of electric light per night, of between 4 88 and 14 1 foot candles intensity (as measured at backs and fronts of the cages), came on heat, copulated, and became pseudo-pregnant, with mammary gland activity Controls underwent no change

(2) Full œstrus was induced in 38–64 days, with typical vulval swelling, successful coitus in 59–70 days, followed by the usual external changes of pseudo-pregnancy

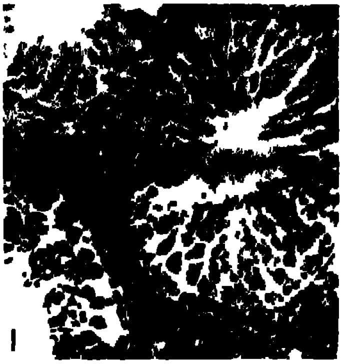
(3) In males, subjected to like treatment, interstitial cells were greatly stimulated This was accompanied by penis and epididymis enlargement to breeding condition, and by increased libido, leading to copulation in 59 days. Spermatogenesis was stimulated to secondary spermatocyte and rare spermatid formation, but no mature sperms were found, even at 71 days

(4) While sexual cycles in female ferrets are conditioned largely, if not completely, by light rations in the visible part of the spectrum, males are much less so, and require some other conditioning factors for complete fertility.

(5) This situation in mammals differs from that in birds, where males are more responsive than females

(6) In the light of parallel reactions of birds and mammals to changes in anterior lobe hypophysis, hormone concentration and balance, it is suspected that there is a common fundamental cause, and that light may act through modifying anterior lobe activity, or susceptibility to it.

(7) A fundamental genetic difference between birds and mammals, correlated with this difference in reaction to anterior lobe hormone and light ration changes, is pointed out.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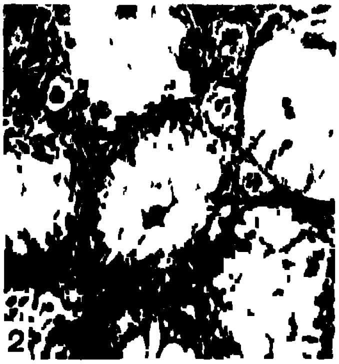
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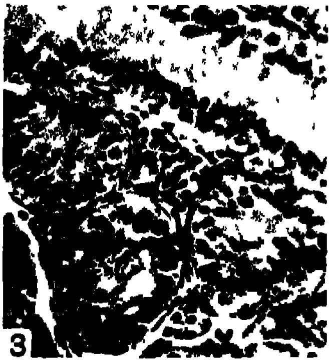
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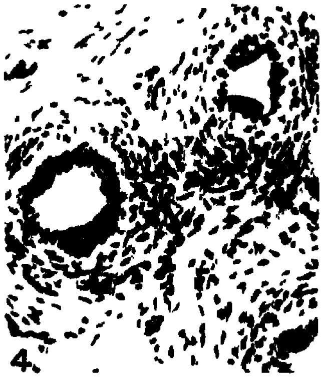
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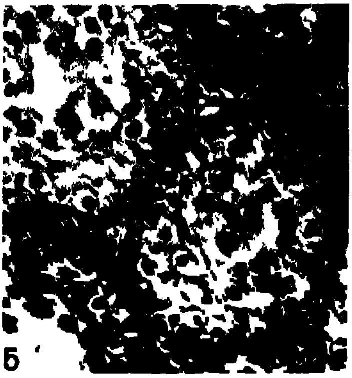
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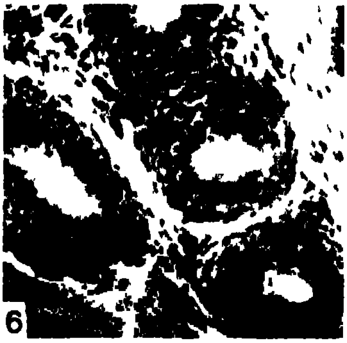
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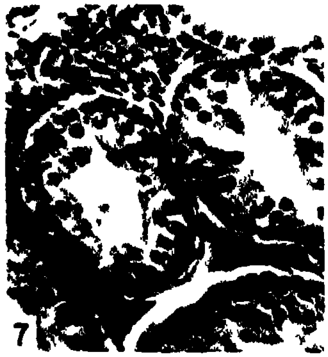
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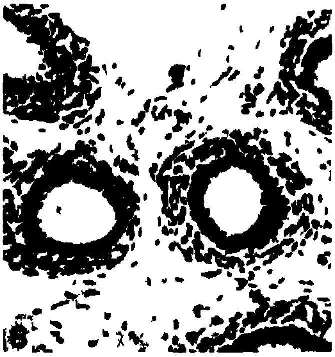
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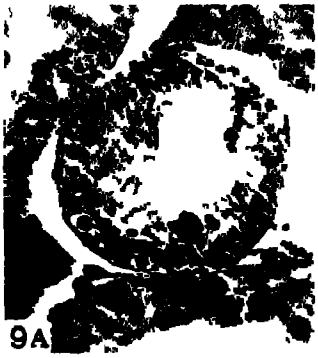
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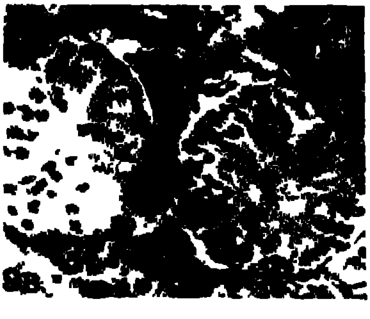
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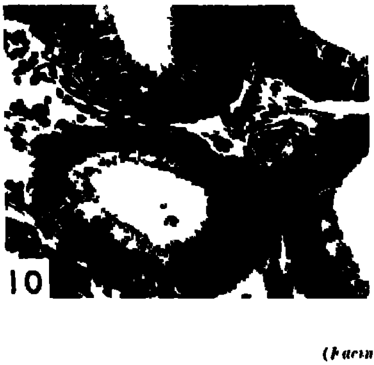
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EXPLANATION OF PLATE.

N.B —Figures are from photomicrographs all taken at the same magnification of about 335 diameters in the first instance and reduced in reproduction.

FIG. 1 —Testis tubules of normal June ferret, in complete spermatogenesis

FIG 2.—Testis tubules of an October ferret, vasectomised the previous March, and copulating six times successfully before being killed.

FIG. 3 —Testis tubules of a normal ferret killed December 12, 1931.

FIG 4 —Epididymis section from normal ferret killed December 12

FIG 5 —Testis tubules of light-treated ferret 1B, died November 18, after 37 days of light treatment

FIG. 6 —Epididymis section of light-treated ferret 1B

FIG 7 —Testis tubules of light-treated ferret 1A Died November 25, after 44 days of light treatment

FIG 8 --Epididymis section from light-treated ferret 1A

FIGS 9 A and B —Testis tubules of light treated ferret, No 2 Killed December 22, after three copulations between December 10 and 21, and 71 days of light treatment

FIG 10 —Epididymis section from light-treated ferret, No 2

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The Formation of Ice in Protoplasm

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[PLATES 14-17]

Though the effect of freezing plant tissues has been studied by various workers (Bachmetjew, 1901, Cameron, 1930, Gortner, 1929, Maximow, 1929, Payne, 1926, Pfeffer, 1903, Stiles, 1930) almost nothing is known of the way in which ice forms within living protoplasm Molisch (1897) seems to have been the first to describe the appearance of minute blocks of ice within the substance of amœbæ frozen under the microscope in a specially constructed cold chamber On thawing, the substance of the amœba had a sponge-like appearance, the spaces representing the places which had been occupied by the ice

When living muscle is thoroughly frozen ice forms between as well as within the fibres, and the relative quantity in the two situations depends upon the rate of cooling, as was first clearly shown by Plank, Ehrenbaum and Reuter (1916)

We have directly followed, under the microscope, the actual formation of ice within and upon the surface of isolated cells and found that the cell membrane can act as a barrier delaying or preventing invasion of the cell by ice, so that the temperature of freezing of the protoplasm may appear to be lower than that of the outside medium

The following cells were used isolated muscle fibres from the sartorius of the frog, fresh water amœbæ supplied from the Zoological Laboratory at Cambridge, and the epidermal cells of the bulb scales of the red onion.

A compound microscope and a micro-manipulating apparatus together with a suitable heating device and a thermocouple were set up in a room at the desired temperature The tissue, however, was prepared in a room at the ordinary air temperature

Portions of muscle fibres, 3–5 mm in length, were transferred directly into a dish of liquid paraffin where they were moved about several times to remove all but a minimum of the surrounding lymph They were then placed on a coverslip in a hanging drop of the paraffin The amœbæ were first placed in a droplet of water on a coverslip and the droplet surrounded by a ring of liquid paraffin enclosing a space of about 6 mm in diameter As much as possible of the water was removed and a drop of liquid paraffin was then deposited so as to make contact with the ring of paraffin and to enclose the water drop The amœbæ thus lay more or less tightly pressed against the coverslip in a minimum amount of water which was enclosed within liquid paraffin In this condition the amœbæ remain alive for hours The onion epidermis was removed from the convex surface of the bulb scales in thin tangential sections which were immersed directly in the oil The tissues and cells prepared in this way were carried on coverslips to a cold room at 0° C where they were kept for varying lengths of time, from 5 minutes to 1 hour, before being transferred to the desired low temperature

The coverslip with the hanging drop was mounted on a chamber on the stage of the microscope The chamber consisted of a glass slide with four brass blocks, 1 cm high, placed at four corners to support the coverslip In this way the chamber was open on all sides to permit the insertion of the various instruments for operation The temperature of the hanging drop was ascertained by immersing in it a 5–6 mm length of a copper-constantin terminal of a thermocouple attached to a galvanometer A microheating device consisted of a small loop of fine platinum wire the ends of which were soldered to the terminals of two copper wires connected with a 2-volt battery and a simple, sliding potentiometer The platinum tip of the heating device and the constantin-copper terminal of the thermocouple were inserted, one on each side of the microscope, between the brass supports of the operating chamber so that they could be approached to or immersed in the hanging drop. When a temperature measurement was actually being made the thermocouple was, of course, within the oil but as close as possible to the cells then under observation A microneedle and a micropipette mounted on a micro-manipulator, projected into the chamber from the front in the usual way.

No difficulty was encountered in securing subcooling without freezing of the cells and of the medium surrounding them This was due undoubtedly to the well known fact that liquids in capillary spaces withstand freezing far more readily than do liquids in larger amounts

The subcooled tissue and the medium surrounding it were caused to freeze, while under observation, in the following way A small mass of ice-snow was deposited on the under surface of the coverslip close to the hanging drop While the temperature of the drop was being determined by means of the thermocouple a minute ice crystal was seized on the tip of the microneedle and carried through the superficial layer of oil of the drop into the film of liquid which contained the cells. In order to freeze the interior of the cells a micropipette containing ice was used The pipette was prepared by filling it with water and inserting its tip into a mass of snow on the coverslip and then immediately removing it The consequent freezing of the water resulted in a minute column of ice projecting from the tip of the pipette

The temperature of the hanging drop during the period of observation was considerably higher than that of the cold room in which the observations were made This was primarily due to the heat from the microscope lamp and from the bodies of the two observers To lessen this rise in temperature a large, cooling filter, filled with dilute glycerol and floating ice, was interposed between the light and the microscope, and the microscope itself was shielded by wrappings of paper sheets and the microscope and manipulator were touched only with mittened hands A low power substage condenser was used but occasionally this was dispensed with, the condensing being done by the concave mirror of the microscope The objectives most commonly used were the Leitz ♮3 and ♮5 A Leitz demonstration eyepiece was used constantly so that all the observations were made simultaneously by two observers.

The contractility of the fibres at room temperature was tested by blowing a small quantity of 1/10 M $CaCl_2$ with a micropipette on the surface of the fibre. This induces a pronounced contraction of a healthy fibre Stimulation with electrodes was tried with indifferent success, possibly because the tips of the electrodes became coated with oil as they were being pushed through the drop to reach the fibre

EXPERIMENTAL

A —*Isolated Muscle Fibres* (*Frog Sartorius*)

1. *Subcooling* —Subcooling within limits does not seriously affect the ability of the fibre to undergo contraction after it has been brought back to room temperature

Some fibres were kept at —6 5° C for 2 hours On being tested with 1/10 M $CaCl_2$ at room temperature the fibres gave a violent contraction Others were kept at —15° C for 3 hours and then brought back to the laboratory. Most of them underwent irreversible contraction during their transference from the cold to the warm room but those which remained extended gave a distinct response with $CaCl_2$.

2 *Surface Freezing*—To obtain surface freezing of the fibres the observations were made in a cold room kept at a temperature of —3° C. ± 0·1° C.

Fig. 1, *a*, Plate 14, shows a fibre with a minimal film of interstitial fluid separating it from the liquid paraffin in which it was immersed When the galvanometer indicated a temperature of — 1 3° C the fibre was touched at one end with a crystal of ice This started the formation of ice flakes which spread over the surface of the fibre from the touched end to the other, fig 1, *b*, Plate 14 Owing no doubt to slight differences of temperature, or to variation in the thickness of the film of fluid, the rate of travel of the surface freezing was found to vary considerably, the rate being sometimes 3–4 seconds and at other times 8-9 seconds for a length of 3 mm No surface freezing could be produced at a temperature above —1 2° C of the hanging drop At —1 4° the rate of freezing was distinctly increased, and at —1 5° C it was rapid

When the preparation was thawed the fibres usually remained uncontracted although occasionally some underwent partial contraction On being brought back to a warm room (approximately 18–20° C) the extended fibres could be made to undergo contraction with $CaCl_2$

A general idea of the consistency of a fibre imbedded in ice and, therefore, as it will appear later, partially dehydrated, is shown in fig 2, *a*, *b*, *c*, Plate 14 This fibre, freshly prepared, was kept at —3° C for half an hour The light was then turned on whereupon the temperature of the drop rose to —1·2° C At this temperature an ice crystal was applied to one side of the fibre and surface freezing slowly extended from the point of application all over the fibre When removing the needle which carried the ice crystal used for seeding, some of the ice on the fibre broke off and was carried away During the manipulation partial thawing took place as the temperature rose for a few minutes to —0·8° C. The temperature then dropped to —1·2° and the freezing slowly extended again The temperature finally became stationary at —1 3° with the entire fibre covered with ice except for one region as shown in fig 2, *a* After 1 hour in this frozen condition the bare region of the fibre was compressed on one side with the point of a microneedle, fig 2, *b*. On removing the needle the bulge slowly diminished, fig 2, *c*, indicating a marked increase in consistency over that which obtains in a muscle at ordinary temperatures. Fifteen hours later the fibre was thawed with no appreciable contraction taking place

The change in form of the superficial ice and the fact that a fibre can undergo contraction while coated with ice (thus partially frozen) is shown in fig 3, *a–c*, Plate 14 Fairly rapid surface freezing was produced at —1·4°, fig 3, *a* The fibre was then left at -2 0° for 3 hours and then re-examined, fig 3, *b* The superficial ice flakes have considerably enlarged and more or less fused together The fibre was then partially thawed but without allowing the greater part of the ice to melt A spontaneous contraction then took place, shown in fig 3, *c*, where one end of the contracted fibre is indicated at *x*

Some attempts were made to determine visually whether the fibre lost water to the exterior during the process of surface freezing With long standing there does seem to be an increase in the total amount of superficial ice but, owing to the fact that the ice crystals tend to fuse into irregular masses, the measurements proved to be too uncertain for comparison However, it is significant that the longer a fibre has been allowed to stand with its surface frozen the less likely can visible internal freezing be induced even at such low temperatures as —5° and —6°

3 *Internal Freezing*—No internal freezing of a fibre was ever observed until the temperature of the drop fell to —1 6° C , and then only when freezing was started *within* the interior, *e g* , by bringing an ice-crystal into contact with the cut end of the fibre or by plunging an ice-tipped micropipette through the sarcolemma

Fig 4, Plate 14, represents a fibre which was brought into a room at —3° C. The hanging drop under the microscope took on a temperature of —1·8° C and the fibre appeared as in fig 4, *a* In this subcooled condition the fibre was punctured with a needle and moved about, but no freezing occurred The fibre was then touched at its cut end with an ice-crystal Surface freezing rapidly spread over the fibre and this was followed, at a distinctly slower rate, by the progressive advance of slender columns of ice through the substance of the fibre Fig 4, *b*, shows the fibre completely frozen internally and externally The rate of advance of the external ice was of the order of 7–8 seconds along a length of 3 mm , while that of the internal ice columns was 10–15 seconds for the same length When the same experiment was made at a temperature of —3° C the rates were much increased, but in all cases the internal freezing was distinctly slower than the external On thawing, the internal ice melted first. The fibre then shortened with signs of internal disintegration into prominent longitudinal fibrillæ Fig 4, *c*, is a photograph after thawing of the fibre in fig 4, *a* and *b* The shortened portion is appreciably thickened and the coarse fibrillar structure is apparent Around this portion is an appreciable amount of exuded water

Fig 5, Plate 15, shows a fibre which had been frozen in a room at —5° C It shows the pointed tips of numerous internal ice columns By slightly warming the drop the columns recede They advance when the temperature is again lowered Fig 6, Plate 15, shows another fibre which had been frozen, thawed and frozen again three times in succession The photograph was taken in the last frozen condition The ice columns are comparatively large and few in number, a condition which occurs after several successive freezings

During thawing, the conical tips of the ice cylinders become rounded and the recession apparently consists in a reabsorption of the liquefied column into the surrounding sarcoplasm Occasionally, the resorption farther along the column is more rapid than at the tip This results in a breaking of the column into one or more spherical and sometimes ovoid droplets which are left *in situ* while the rest of the column continues to recede The droplets which are left behind diminish and vanish after a second or two

Fig 7, Plate 15, shows another fibre with the tips of internal ice columns If the advance is slow the columns may not extend beyond a certain distance When this occurs it was found, by probing with a needle, that the unfrozen portion of the fibre was very stiff This suggests that this part of the fibre had become dehydrated by the loss of water to the advancing columns

The highest temperature at which internal freezing could be induced was —1 6° C At this temperature the advance of the columns of ice was slow At —1 8° there was a distinct acceleration Lowering of the temperature also resulted in a progressive increase in number with diminished diameter of the ice columns The fibre shown in fig 8, Plate 15, was internally frozen at a temperature of —5° C The ice columns are numerous and slender and it is of interest to note that they run spirally This is due to the fact that the fibre had been twisted before freezing There seems to be no doubt that the formation of the longitudinally arranged ice columns is an indication of longitudinally arranged solid elements in the fibre When the fibre is twisted the solid elements or myofibrillæ are correspondingly twisted and, therefore, the formation of ice in the fluid interstices between the fibrillæ assume the same course

A significant feature of the internal freezing is that the mere presence of ice outside the fibre does not produce internal freezing If a fibre is touched with an ice-crystal at some point along its middle, the surface freezing spreads from the point in all directions, and it is not until the ice has reached the cut ends of the fibre that internal freezing starts at those two ends and spreads inward If the surface of the fibre is torn at a spot in advance of the forming external ice, then internal freezing starts at the torn spot so soon as the external ice reaches it Another method of producing internal ice is to puncture a fibre with an ice-tipped micropipette The internal ice columns start at the tip of the pipette and spread in two directions through the fibre

If a subcooled fibre, cut at both ends, is inoculated with ice at one end the external ice will reach the other cut end considerably in advance of the ice columns travelling along the interior Inoculation of the interior at this end may then take place with the result that a new set of internal ice columns is generated They travel down the fibre to meet those which had been started at the other end Fig 9, *a*, Plate 15, shows this in a fibre internally frozen at —6° C with ice columns which have been generated at both ends of the fibre Fig 9, *b*, Plate 15, shows, under higher magnification, several ice columns, *x*, with their tips extending downward in the figure These columns had been started by the arrival of the external ice at the cut end of the fibre opposite to the end where the original inoculation had been made

Occasionally an internally frozen muscle fibre was too dense to permit visibility of the interior In such cases partial thawing brought the longitudinal columns into view, as if intervening, fine crystals had thawed out

Fig 10, Plate 15, is a photograph of a fibre which had been caused to freeze internally by seeding at one cut end The ice columns advanced rather slowly and stopped before reaching the other end of the fibre After several hours in the low temperature room the preparation was partially thawed and then refrozen The ice columns receded during the thawing and when they advanced again they did not reach their original lengths The preparation was left in the low temperature room till the next day, when the photograph was taken The photograph shows the ice columns in the refrozen condition Beyond the tips of the columns can be seen delicate splits in the fibre caused by the previous advance of the ice columns during the first freezing This preparation demonstrates the dehydrating action of internal freezing When the freezing is allowed to occur at a slow rate water evidently is withdrawn from the fibre in advance of the forming ice columns The result is that the columns do not extend the full length of the fibre and the phenomenon becomes accentuated after thawing and re-freezing. Incidentally the photograph also shows the swelling effect on the fibre when the interior is frozen.

Fibres in which internal freezing was complete were always found to be dead on thawing (see later, p. 347).

B.—*Freezing Experiments with the Fresh-water Amœba*

Movement of the animal as a whole ceases when an amœba is subcooled to −4° or −5° C., but slow streamy movements still continue and they cause slow but continuous changes in shape.

Agitation with a microneedle causes a back and forth flow of the granules, but the flow is passive and stops as soon as the needle is held motionless. Occasionally, a hyaline zone slowly appears beneath the periphery of the amœba, indicating a gradual movement of the granules. The pellicle can be pulled out into a long strand which slowly returns when released.

A peculiar feature in these subcooled amœbæ is an apparent stiffening of the pellicle and of the vacuolar membranes. The contractile vacuole could be pinched into two spheres, an operation which is very difficult in an amœba at ordinary temperatures when such an operation usually results in the bursting and dissipation of the vacuole. The readiness with which reformation of a membrane occurs is also shown by the ease with which a pseudopodium could be cut off. The separated portion quickly rounds up while the rest of the amœba recovers with no loss of its contents.

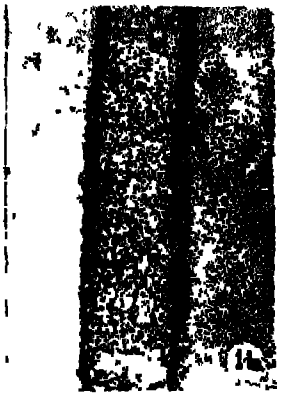
Fig. 11, *a*, Plate 16, represents an amœba in subcooled water at −0·6° C. Fig. 11, *b*, Plate 16, is the same amœba after the surrounding water had been frozen. The form of the amœba in both conditions is practically the same. However, slight changes in shape do occur. When the external freezing process is slow, *e.g.*, at −0·3° C., and is started at some distance from the amœba, the advancing borders of the growing, flat cakes of ice move more slowly the closer they approach the amœba and they stop before coming into contact with it. It is not until the amœba is surrounded with ice that the narrow zone of water about it freezes. When the preparation is made with a very thin film of water around the amœba the ice is fragile enough to be indented and pierced with microneedles. It can be then shown that the interior of the amœba is still fluid and the cytoplasmic granules can be moved about by compressing the amœba. This was done with the temperature of the drop at −0·5° C., and as long as the surface membrane of the amœba was not broken the interior remained fluid although the amœba was completely embedded in ice. Several amœbæ were kept in this condition with the temperature of the drop at −1·4° C for 2 hours The preparation was then thawed and brought to room temperature at 18° C, when the amœbæ regained their pseudopodial movements.

Internal freezing was produced by inserting an ice-tipped pipette into the interior of the amœba when the temperature of the drop was at −0·8° C or below The ice which formed spread in all directions from the tip of the pipette as fine, feathery crystals quite in contrast to the definitely directed ice-columns which develop in a muscle fibre The spread was rapid and in several cases was observed to encircle and not immediately to penetrate the contractile vacuole and the nucleus Freshly frozen amœbæ are opaque when viewed with transmitted light It was a striking picture to prick one amœba after the other with an ice-tipped pipette and to see the punctured amœbæ flash out dense and black in contrast to the unpunctured amœbæ in the field After several minutes the opacity of the frozen amœbæ diminished, as the feathery ice crystals merged into transparent blocks which pushed the granules of the amœbæ into spaces between them Figs 12, *a*, *b*, *c* and *d*, Plate 16, are from photographs taken successively to show this change A very shallow drop of water containing four amœbæ and enclosed in liquid paraffin at −0·8° C. was made to freeze into a thin sheet of ice by inoculating the water with an ice crystal One of the amœbæ was punctured with an ice-tipped pipette The result is shown in fig. 12, *a*, in which the frozen amœba can be distinguished from its unfrozen neighbours by its darkened appearance Several minutes later, fig 12, *b*, another amœba on the right of the figure was likewise punctured with the same result. The figure also shows the previously frozen amœba, *x*, in which the ice had changed into transparent blocks giving the amœba a spongy appearance Ten minutes later the preparation was thawed. The resulting disturbance swept most of the material out of the field so that fig. 12, *c*, shows only one of the unfrozen amœbæ still intact and the spread-out, coagulated mesh of one of the amœbæ which had been internally frozen The preparation was then carried to a warm room where the intact amœba recovered, fig 12, *d* In the lower part of the figure the remains of the frozen amœba can still be seen

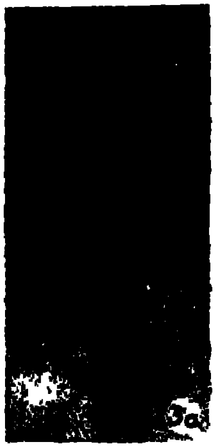
C.—*Epidermis Cells of the Bulb Scales of the Red Onion.*

Strips of the single cell-layered epidermis were removed from the bulb scales of the common red onion and immersed in hanging drops of liquid paraffin. A thin film of sap lay between the tissue and the surrounding oil. After a sojourn at 0° C. the preparation was carried to a room at −6° C. when the temperature of the hanging drop was probably −3° to −4° C. After 5–10

1a


1b

2b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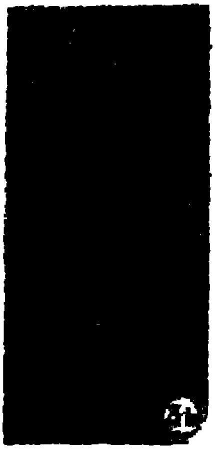
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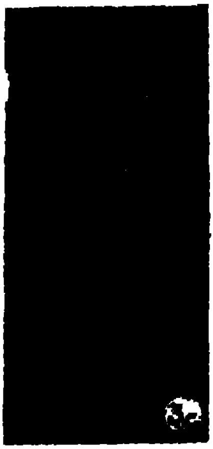
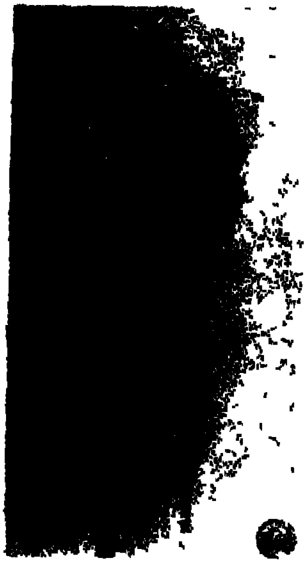
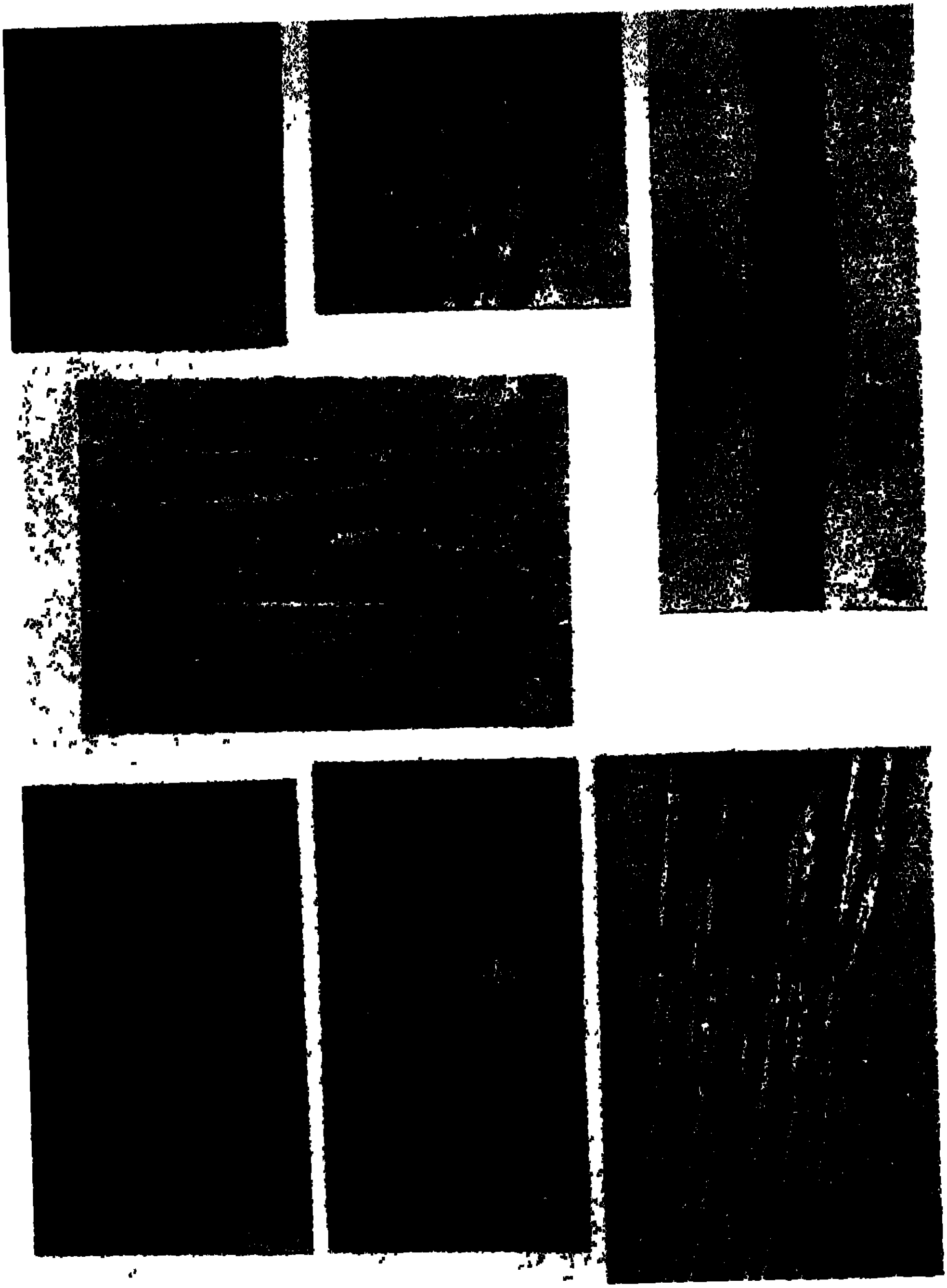
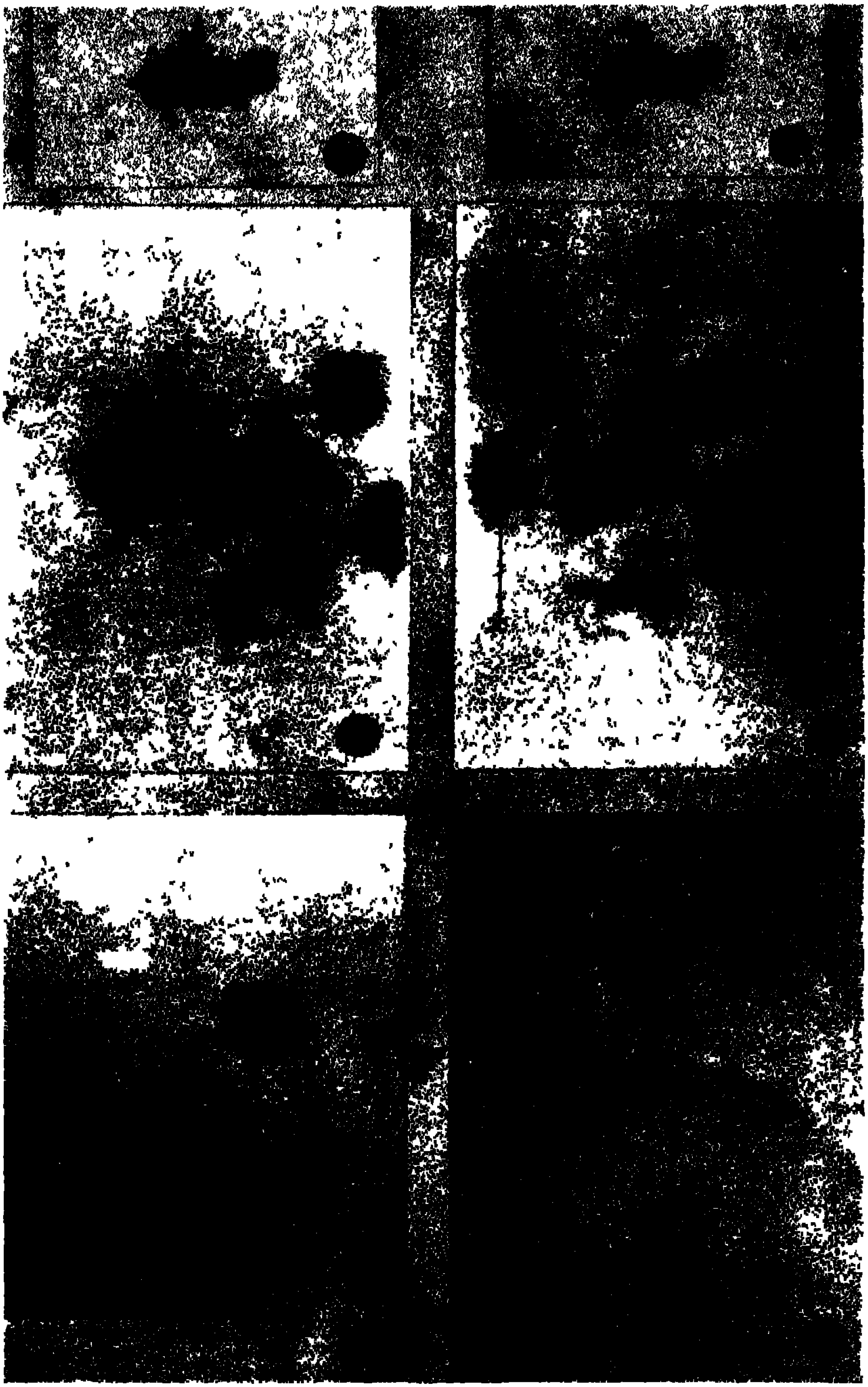

3c

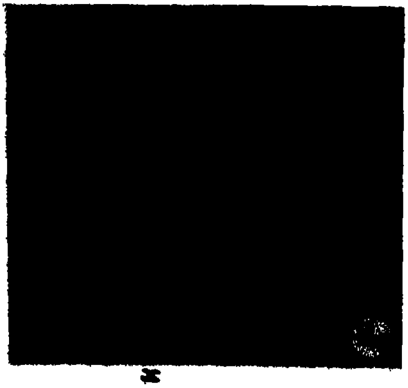
2c

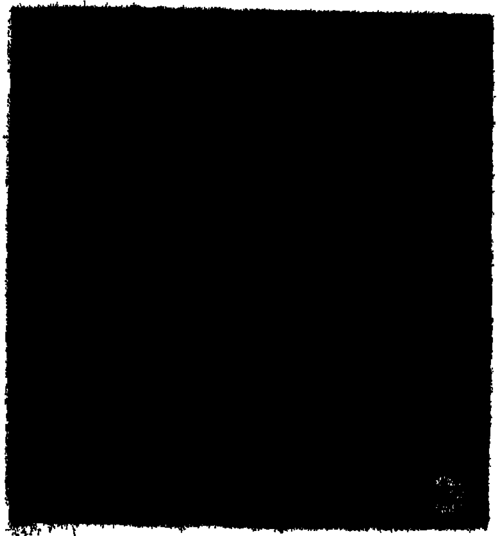
4a











minutes the strip of tissue was touched with an ice-crystal This resulted in the freezing of the sap covering the tissue and lying within the dead cells, while the cells with living protoplasts remained unfrozen.

In the room at −10° C. a peculiar phenomenon frequently occurred There was first the usual surface freezing and the coarse-meshed freezing of the contents of dead cells, while the presumably living cells with coloured sap retained their normal appearance After a brief interval fine ice-crystals suddenly appeared in one purple cell and then in another in successive flashes until all the cells were involved On thawing, many of the cells retained their purple colour, indicating that the vacuoles were still intact On the other hand, a sojourn of 2 hours in the frozen condition resulted in a coarsening of the colourless ice-crystals, and the pigment appeared more concentrated and frequently in isolated areas within the cell It was not ascertained whether the ice-crystals formed within or outside the vacuoles The impression gained was that both occurred The separation of the vacuole into smaller, densely pigmented vacuoles, suggests that the ice was formed outside the vacuole and had increased in size by the extraction of water from the vacuole It is well known that plant-cell vacuoles or tonoplasts will divide into several vacuoles during plasmolysis, and will reunite upon deplasmolysis, Küster (1929). We have observed something similar on freezing When frozen to −10° C the cytoplasm and nucleus disintegrated, but the tonoplast, which is more resistant than the protoplast, plasmolysed and often divided into several vacuoles with intact membranes When thawed, the vacuoles often reunited with complete deplasmolysis Subcooling to a temperature of −10° C. for hours did not induce plasmolysis. This occurred only when the tissue was made to freeze.

When the freezing was relatively slow, the ice was observed to form between the cellulose wall and the tonoplast As the ice increased in amount, the wall of the tonoplast was pushed inward and the pigment of its contents became progressively deepened in colour When the tissue was thawed the tonoplast frequently regained its former volume

Figs 13, *a*, *b*, Plate 17, shows the cut end of a strip of onion epidermis in liquid paraffin. In fig 13, *a*, the rounded end of a tonoplast can be seen protruding from a cut-open cell along the edge of the strip The preparation had been frozen at a room temperature of −10° C and the ice can be seen as whitish areas around the dark coloured vacuoles. The cell with the protruding vacuole contains ice bordering the inner end of the vacuole which appears to have been partially pushed out of the cell by the ice formation. In fig. 13, *b*, is shown the protruding outer end of the same vacuole being pulled with the tip of a microneedle in the liquid paraffin

Fig 14, *a*, Plate 17, represents a surface view of onion epidermis before freezing The majority of the cells contain intact vacuoles with purple sap Fig 14, *b*, represents the same region after freezing in a room at −10° C The partially shrunken vacuoles are darker, presumably because of the more concentrated sap, and the lighter zones about them represent the ice The cell marked *x* in these two figures and the group of seven cells surrounding it are shown enlarged in fig 14, *c* Note the extent to which the vacuole has been shrunken and deformed by the ice surrounding it The surrounding cells have not been plasmolysed to the same extent They appear in better focus in fig 14, *b*

Fig 14, *d*, represents the region shown in fig 14, *b* and *a*, after the preparation had been thawed Cell *x* has not recovered to the extent of the seven surrounding cells By comparing other cells in the three figures one may note the extent to which freezing may occur with subsequent recovery For example, the cell *y* underwent considerable freezing with apparently complete recovery

On thawing, the ice melted to form droplets of water between the cellulose wall and the shrunken protoplast Within 2 minutes this water had disappeared and the protoplasts regained their original volumes In places the vacuole was broken and the cytoplasm appeared as coagulated, shrivelled shreds together with the coagulated nucleus in a faded cell In other places the vacuole or tonoplast remained intact with the shrunken and dead protoplast pressed against the cellulose wall It is significant that the coloured vacuoles, when shrunken in the frozen condition, were much darker, *i e*, more concentrated with pigment, than in the expanded condition before freezing and after thawing

Discussion

Three broad conclusions emerge from these observations —

(1) That the plasma membrane of the living cell acts as a barrier against internal freezing,

(2) That internal freezing, the freezing of the protoplasm itself, kills, and the cell plasmolyses on thawing On the other hand,

(3) Living protoplasm can be subcooled to an extraordinary extent without being killed, isolated muscle fibres were in fact subcooled to −15°.

Jensen and Fischer (1910) and Weigmann (1929) state that the minimum survival temperature for frog muscle is in the neighbourhood of −1·4°.

Moran (1930), however, in a detailed study found that when the muscle was frozen below −2° it was irreversibly injured Since we were never able to induce internal freezing even by inserting ice into the interior of a fibre, unless the external temperature was −1 6° C. or below, it is possible that Moran's critical temperaure is the temperature at which internal freezing can take place

It is, however, important to note that the morphological picture of a muscle fibre is not necessarily disorganised by inducing internal freezing with an ice-tipped pipette so long as the freezing has not proceeded too far or lasted too long We have frequently caused thawing to take place by warming a fibre while the ice-columns were still advancing within the fibre and noticed the temperature of melting of the columns and the resorption of the water leaving the fibre normal in appearance with uniform transverse striæ and no sign of longitudinal splitting On the other hand, a fibre which has been completely frozen internally and left in that condition for some time, will, on thawing, shorten and thicken irreversibly, and give the appearance of cytolysed muscle with prominent bundles of longitudinal fibrillæ We have never observed that cold or freezing ever produces contractions which can in any sense be considered a physiological phenomenon as has been claimed recently by Hürthle (1931), who speaks of a "Gefnerungsreiz" A fibre which has been subcooled to a temperature of −10° C or below and then is suddenly brought into a warm room will sometimes undergo considerable shortening, but such a contraction is always an irreversible one An internally frozen fibre on thawing always contracts and undergoes cytolysis with loss of water

So long as the plasma membrane remains intact the interior of a cell can be subcooled below the temperature of its freezing point The critical temperature of tissues therefore appears to be the one at which the barrier breaks down so as to permit the external ice to inoculate the interior

In observations on ice formation in subcooled liquids one is struck by the absence of any pushing tendency on the part of the advancing ice-crystals The advance must occur by the congealing of the water that is present in advance of the crystal. Hence, it is reasonable to suppose that the steady and uniform advance of the longitudinal ice-columns during the internal freezing of a muscle fibre is along uninterrupted fluid interstices between more solid constituents. It follows, therefore, that the evidence presented in this paper argues against the existence of transverse membranes, stretching across the fibre in striated muscle and is in favour of the existence of longitudinal myofibrillar structures

In the amœba the spreading at random of fine, feathery crystals indicates the lack of a definite structure Future investigation will show to what extent this micro-method of inducing internal freezing will throw light on structural elements within a greater variety of cells

Since all our operations dealt with volumes of capillary dimensions the well-known effect of capillarity in depressing the temperature of the freezing point must be reckoned with

Müller-Thurgau (1880–1886) found that the freezing-point of a gross quantity of expressed sap is higher than that of the sap when enclosed in the vacuoles of the plant cells He compares this phenomenon with the fact that the freezing-point of water is depressed by 0 2 to 0 3 of a degree when it is held in the interstices of filter paper

In like manner the difference which we found between the temperature of the cell interior and that of its external medium, already itself of capillary dimensions, may be explained in part by assuming the existence of still finer capillary spaces within the cell This must be true for the muscle fibre in which internal freezing takes the form of parallel bundles of slender ice-columns through the length of the fibre Such structural conditions in the intact fibre may account for the fact that the freezing-point observed by us was fully one degree lower than the freezing-point generally agreed upon for the expressed juice of frog muscle, namely, —0 43° C, Hill and Kupalov (1930)

In a subcooled amœba there is no complete cessation of streaming The granules in the cytoplasm slowly shift their position and there is a gradual change in contour This slow activity is accompanied by a suggestion that heat is being evolved by the subcooled cell (at a temperature of —0 3° C), ice formation in the external medium is definitely slower when the advancing edge comes close to the cell

In the epidermal cells of the onion the presence of a large central vacuole with watery contents offers a good illustration of the dehydrating property of the freezing process by plasmolytic action In order that freezing of the vacuolar fluid may occur the external ice must pass through two barriers, the plasma-membrane surrounding the protoplasts and the inner membrane or tonoplast which surrounds the vacuole Even when the protoplast is destroyed the vacuolar membrane or tonoplast may still remain intact and prevent freezing of the vacuolar contents The rigid cellulose walls offer an interesting contrast to the plastic protoplast in the penetration of the freezing process The cellulose wall itself acts only as a temporary obstacle while the protoplast shrinks down on the diminishing vacuole, and no internal freezing takes place until the protoplast is destroyed

In conclusion, the factors which prevent the freezing of the interior of a living cell may be summarised as follows (1) the retardation effects of capillarity due to the microscopic dimensions of the cells, (2) dehydration which increases the total and balanced salt concentration of the interior, (3) the evolution of heat through metabolic activity, and (4) last but not least, the existence of a plasma-membrane which effectively prevents the transmission of the freezing process to the subcooled interior even when ice is in contact with its external surface

A critical temperature exists below which these factors are overcome, the plasma-membrane then breaks down, the integrity of the protoplasm is thereby lost and death ensues When the subcooled interior of a cell has been artificially frozen by the introduction of an ice-tipped micropipette the plasma-membrane may be still intact, but apparently this condition does not last long

Summary

A *Muscle Fibres of Frog*—(1) Cold alone does not kill, even subcooling to —15° C

(2) Ice formed on the outside of fibres at about —1 2° C and inside fibres at —1 6° C On thawing, the internal ice melted first, but a fibre could be subcooled to —10° C without internal freezing, unless the sarcoplasm was brought into direct contact with ice by breaking through the sarcolemma which therefore acts as a barrier

(3) Ice formed inside the fibre was always in longitudinal columns, which grew steadily from the point seeded There was no indication of transverse membranes

B *Amœba*—(1) Amœbæ can be subcooled, without death taking place, to at least —5° C, even when the external water is frozen Internal freezing occurred only at —0 6° C and below, sometimes spontaneously, and always when ice was brought into direct contact with the interior

(2) The barrier against internal freezing is the cell membrane Most, if not all, of the internal water can be frozen

(3) Internal freezing killed the animals The cell contents, on thawing, formed a coarse mesh

C *Epidermal Cells of the Onion Bulb Scale*—(1) Strips of tissue were frozen between —7° and —10° C Ice appeared readily between the cellulose wall and the protoplast with plasmolysis of the latter, which, however, was reversed upon thawing

(2) The ice sometimes broke the outer membrane of the protoplast but the vacuole or tonoplast sometimes remains intact, in which case the tonoplast deplasmolyses on thawing

(3) Ice was not definitely seen to form within the vacuole but the general impression was obtained that it did so form in some cases

APPENDIX

The Freezing of Cytolysed Muscle Fibres

The experiments in the main body of this paper were all made on living material, and the muscle fibres used reacted to stimulation after exposure to the various low temperatures and within the time limits of the experiments

The following experiments on muscle fibres were done in order to determine whether dead muscle fibres differ from the living in the way in which freezing takes place

Pieces of fresh muscle from the sartorius of the frog were placed for several hours in distilled water Microscopic examination showed the fibres as a bundle of prominent fibrillæ enclosed by a sarcolemma-like membrane These fibres were isolated in liquid paraffin, freed of surplus aqueous fluid, and mounted in the usual way for manipulation

A hanging drop of oil, containing several cytolysed fibres, was brought to a temperature of —2 4° C Their surfaces were then touched with an ice-tipped micropipette Freezing spread rapidly over the surface When the cut ends were reached internal ice columns formed and proceeded at a much slower rate through the interior

When the temperature of the hanging drop was at —5° C seeding a fibre produced a very rapid surface freezing together with the formation of internal ice columns. The rate was too rapid to observe how the internal freezing occurred

We may conclude, therefore, that the sarcolemma of a cytolysed muscle fibre may serve as an obstacle to internal freezing but probably not as efficiently as that of a living cell

EXPLANATION OF PLATES

PLATE 14 —Isolated muscle fibres from sartorius of frog in liquid paraffin

FIG 1 (*a*) —Fibre at -1 3° C, unfrozen. (*b*) Superficially frozen after being touched with ice

FIG 2 —(*a*) Fibre superficially frozen at —1·2° C and left overnight at —3 0° C (*b*) Bulge produced on naked portion of fibre by compressing with microneedle (*c*) Bulge gradually disappearing after removal of needle.

FIG 3 —Fibre at -1 4° C (*a*) Superficially frozen. (*b*) After 3½ hours in frozen condition ice has collected in larger flakes (*c*) Contraction of fibre after partial thawing Note tip of fibre at *x*

FIG 4 —Fibre at —1 8° C (*a*) Unfrozen Fibre was punctured with no effect (*b*) Both externally and internally frozen after inoculation with ice (*c*) Irreversible shortening upon thawing with exudation of aqueous liquid.

PLATE 15 —Muscle fibres with internal ice-columns.

FIG 5 —Fibre internally frozen in room at —5° C Lower portion of fibre focussed to show numerous internal ice-columns with tapering tips Upper portion of fibre not yet internally frozen and focussed to show external ice flakes

FIG 6 —Fibre internally frozen in room at 6° C for third time and showing tips of few large ice-columns

FIG 7 —Fibres internally frozen in room at —6° C

FIG 8 —Twisted fibre with internal ice-columns conformed to twist

FIG 9 —(*a*) Internally frozen fibre in room at —6° C Upper portion showing downwardly directed ice columns *x*, due to inoculation from upper cut end after spread of surface freezing had reached it (*b*) Detail at high power to show the downwardly directed ice-columns at *x*

FIG 10 —Muscle fibre internally frozen in room at —3° C

PLATE 16 —Fresh-water amœbæ in thin film of water surrounded by liquid paraffin

FIG 11 —(*a*) Amœba in subcooled water at —1 0° C (*b*) Same amœba with little change in contour after the water surrounding it was frozen

FIG 12 —(*a*) Four amœbæ embedded in a thin sheet of ice in oil at - 0 8° C Amœba at left in photograph has just been frozen internally by puncturing with ice-tipped pipette, hence its darkened appearance (*b*) Same field 5 minutes later taken just after amœba at right has been similarly frozen Note, at left of photograph, that amœba *x*, frozen 5 minutes before, is now sponge like in appearance, due to gradual fusing of its ice into translucent blocks (*c*) One of unfrozen, intact amœbæ and coagulated remains of frozen amœba in thawed water at —0 8° C (*d*) Ditto, brought into warm room where unfrozen amœba has recovered. Remains of frozen amœba in lower part of photograph

PLATE 17 —Epidermal strips of Red Onion in liquid paraffin

FIG. 13 —Tissue frozen in room at —10° C Note ice (clear zones) between cellulose wall and dark (red sap) vacuoles of cells (*a*) Cut edge of strip with tonoplast protruding (*b*) Protruding part of tonoplast deformed with microneedle

FIG 14.—(*a*) Surface view of epidermis subcooled in room at 10° C Note cell *x* surrounded by a group of seven cells (*b*) Ditto, after freezing (*c*) Detail of former, with cell *x* in centre, under higher magnification to show plasmolysed vacuoles surrounded by ice (*d*) Same region after complete thawing Some cells are fully deplasmolysed Others were too much injured by the freezing and underwent only partial deplasmolysis, *e g* cell *x*

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The Combination Curves, Hydrogen Ion Regulating Power and Equivalents of Ovalbumin

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The general nature of the combination curve of albumin with acids and alkalies has been stated by Loeb, and is based on titrations of Hitchcock and Cohn (1925) also Bugarszky and Liebermann (1898).

Some preliminary results obtained by one of us showed a marked difference in the 0·1 per cent. solutions on the alkaline side, and difficulty in reproducing the results with 1·0 per cent. solutions. Many more experimental points were required in order to confirm or dispute the differences between albumin and gelatin, which appear to follow from Loeb's smoothed curves, for 1 per cent. solutions; Harris (1925) dealt with some of these differences.

Some points on the acid side of the isoelectric point, *p*I, were obtained by Sørensen, (Miss) Hoyrup, Hempel and Palitzsch (1917), and from these, by a refined use of the equations for homogeneous equilibria, curves were constructed which intersect at nearly the same point, namely, that at which there is no excess either of NH_3 or H_2SO_4 in solution. By this means Sørensen and his co-workers obtained the following constants :—

(1) The isoelectric point, at $(H^+) = 1·57 \times 10^{-5}$ $pI = 4·8$.

(2) The point at which all albumin is "free"

$[H^+] = 1·3 \times 10^{-5}$, natural $p_H = 4·9$.

Following a common practice, we have reckoned additions of acids and alkali from a zero at $p_H = 4·7$ to $4·8$.

Experimental

The metre bridge potentiometer, hydrogen electrodes and calomel half-cells were calibrated and tested in the usual manner, and also by titrating against gelatin. The results were in agreement with those previously obtained, Prideaux (1931). The ovalbumin was prepared by the methods of Hopkins and Pinkus (1898) as modified by Sørensen and co-workers (1917). The crystalline product was purified by dialysis for one or more weeks until the ammonium sulphate was reduced to a low concentration. Loeb's preparation contained 0·001 to 0·002 equivalents of ammonium sulphate, while our 1 per cent. solutions contained 0·0001 to 0·0025 equivalents of ammonia per litre This was determined by a modification of van Slyke's aspiration method (NH_3 in urine). Air was aspirated through a mixture of 5 ml. of the albumin, 3 drops of caprylic alcohol, and 20 ml of 10 per cent K_2CO_3 solution, then through 20 ml of standard, 0 05N, H_2SO_4 The ammonia was estimated by titrating the sulphuric acid with alkali and also by the Nessler method Some stock solutions were dialysed first against distilled water, then against N/100 or N/150 acetic acid for periods of 2 to 4 weeks, the ammonia thus being reduced to 1×10^{-4} g per litre, or to less than 6×10^{-6} equivalents per litre

The albumin content of the stock solutions were determined by —

(1) Evaporation to constant weight at 95°–100°
(2) A special Kjeldahl method (Sørensen and others, 1917, *loc cit*)
(3) The refractivity

The factor 6·4 was used to convert Kjeldahl nitrogen into dry albumin For example, a stock solution contained 1·83, 1 84, 1 86 per cent protein by evaporation and 1 835 per cent by Kjeldahl The results of the titrations are given in Tables I to IV A few results of Loeb which have been corrected for alkali blank are included in Table II

The results of all titrations are expressed in a uniform manner as cubic centimetres of 0·1 N acid or alkali added to 1 g of albumin whether this is present in 1·0 or 0 1 per cent solution The acid and alkali additions in solutions of low or high p_H are corrected in the usual manner, by the potentiometrically determined acid and alkali blanks according to Atkin and Douglas (1924).

The curves, fig 1, resemble those of gelatin in the greater steadiness of the acid titrations as compared with the alkaline ones The differences between the two curves will be discussed in detail

Acid Titrations

The results of all series appear to be in satisfactory agreement up to and slightly beyond p_H 5 0, Table I Any given addition of acid defines the p_H within about ±0 2 A slight downward turn which appeared below p_H 3·0 is chiefly apparent in the "denatured" values, and was not found in pure albumin. The lowest p_H of 2·3 (off the curve) corresponds to the large acid addition of 16·4 c c (24 0 c c uncorrected), D, Table I Since there is no inflection it is impossible to define an acid saturation capacity for albumin. The basic groups are progressively opened up as p_H diminishes This change is largely reversible since it is possible to return along the acid titration curve.

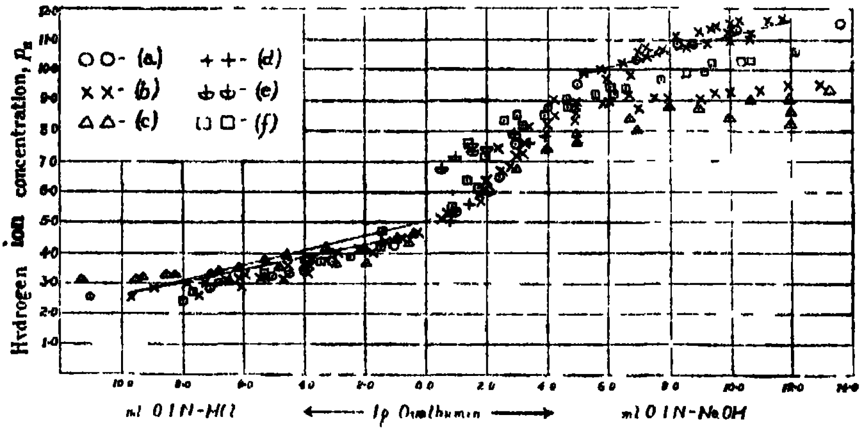

FIG 1—The Combination of 1 0 and 0 1 per cent Albumin with Acid and Alkali

⊙ ⊙ Loeb, 1 per cent in acid and alkaline solution
× × Authors, 1 per cent solutions A, B, C, D
△ △ Authors, 0 1 per cent solutions
+ + Hendrix and Wilson, with quinhydrone electrode, natural albumin
♁ ♁ Hendrix and Wilson, heat coagulated ovalbumin
▣ ▣ Authors, 1 0 per cent solutions, alcohol denatured, and also commercial dried albumin

Tables I and II—Stock Solutions dialysed A, against distilled water for 1 week N/100 acetic acid for 4 weeks, and finally against distilled water for 1 week, C, against distilled water for 18 days, D, against distilled water for 5 days, against N/150 acetic acid for 2 weeks, and finally against distilled water for 3 days

Residual ammonia A, 0 0001 g equivalents per litre, B, 0 002 g equivalents per litre, C, 0 0003 g equivalents per litre, D, 0 00015 g equivalents per litre

1 per cent solutions		Acid titrations 25° C			
A		C		D	
c c 0 1N acid to 1 g albumin.	p_H	c c 0 1N acid to 1 g albumin	p_H	c c 0 1N acid to 1 g albumin	p_H
0 3	4 68	0 44	4 55	0 22	4 6
0 9	4 5	0 81	4 5	0 65	4 4
2 1	4 1	1 30	4 4	1 09	4 25
3 8	3 6	2 17	4 1	1 74	4 05
5 4	3 2	2 94	3 95	2 20	3 85
7 1	2 8	3 69	3 8	2 96	3 65
7 4	2 6	4 46	3 6	3 84	3 85
		5 17	3 4	4 6	3 2
		5 92	3 3	6 0	2 9
		6 58	3 13	7 1	2 7
		7 16	3 05	9 63	2 5
		7 86	3 0	13 1	2 4
				16 4	2 3

0 1 per cent solutions		Acid titrations 25° C			
A		C		D	
c c 0 1N acid to 1 g albumin	p_H	c c 0 1N acid to 1 g albumin	p_H	c c 0 1N acid to 1 g albumin	p_H
0 42	4 6	1 09	4 4	0 1	4 6
2 80	4 25	2 18	4 25	0 47	4 4
4 80	3 6	3 26	4 1	1 20	4 3
5 50	3 2	4 45	3 95	2 10	4 05
6 40	3 0	5 30	3 75	2 30	3 8
7 10	2 9	6 10	3 5	2 85	3 7
13 7	2 8	7 0	3 35	4 0	3 6
		8 5	3 3	4 84	3 55
		9 3	3 2	6 8	3 4
		9 5	3 05	8 2	3 25
				11 38	3 05
				17 43	2 85
				25 3	2 7

The combination curve in 0 1 per cent solution is slightly more variable, and lies above that of the 1 0 per cent solution showing the normal effect of increased hydrolysis

It has been remarked by Harris (1925) that the albumin curve is slightly steeper between p_H 4 6 and 3 6, the gelatin in this region combining with about twice as much acid as the albumin. Our curves confirm this in a qualitative manner, but we find that gelatin combined with 3 9 c c of acid and albumin with 3 6 c c * This much smaller difference does not permit one to discriminate between the two proteins in the manner suggested by Harris, but another possible method of discrimination alluded to by him seems more promising, namely, the absence of inflection in the albumin curve at p_H 2 6 To bring a 1 per cent solution of gelatin to this point requires 8 0 c c of acid, while a 1 per cent solution of albumin requires 10 c c (corrected for blank) Incidentally we have here a method for distinguishing between fresh and denatured albumin (see figure)

Alkaline Titrations —Considerable differences in p_H corresponding to the same amounts of alkali added begin to appear at about p_H 6 0 and are more conspicuously displayed on the curve at about p_H 8 0 after the inflection has been passed In some series the rate of increase falls right off here, and may be, for example, only 0 5 units between 5 and 13 c c of alkali combined, *cf* A and B, Table II We have not been able to connect this behaviour with the mode or length of dialysis of the solution, and it would appear to depend upon the formation of metaprotein quite early in the titration, while the alkalinity is still low, and may possibly be connected with the age of the eggs originally used It is known that the natural p_H of eggs undergoes rapid change on keeping, and it is more difficult to obtain crystalline albumin if the eggs are not absolutely new laid

It is possible that such ovalbumin is more readily denatured Attention has been called to the greater buffering power and lower p_H values of denatured albumin by Wu and Chen (1929)

If the normal curve of the crystalline protein is that of Loeb, then the highest p_H values may be taken as evidence of purity With solution D we obtained some points which lay even higher

In none of these curves, high or low, is there any sign of an inflection. Albumin, therefore, differs from gelatin in showing no sign of saturation with alkali, and consequently no acid equivalent of albumin can be obtained by the ordinary method

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* Over the greater part of the combination curves the positions of gelatin and albumin are reversed as is shown later

Table III —Stock solutions as in Table I Stock solutions A, C, and D dialysed against acetic acid , B, against distilled water 25° C

Loeb (with blank correction)		A		B		C	
c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H
5 0	9 5	0 8	5 0	4 2	9 0	7 0	8 6
6 75	10 3	1 8	5 7	5 15	9 85	9 0	9 0
8 25	10 8	2 8	6 8	5 7	10 07	11 0	9 3
10 3	11 3	3 2	7 25	6 5	10 25	13 0	9 5
13 5	11 5	4 0	7 8	7 13	10 4		*(9 35+ 0 10)
		5 0	8 6	7 83	10 6		
		6 0	8 85	8 52	10 7		
		7 5	9 05	9 26	10 8		
		9 5	9 15	10 0	10 85		
		10 0	9 2	10 74	10 9		
		12 0	9 25				
		15 0	9 35				
		18 0	9 55				
			*(9 45+ 0 10)				

D		D		D		D	
c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H
2 0	6 0	1 0	5 3	4 0	8 4	3 0	6 9
3 0	6 8	2 0	6 0	6 0	9 7	4 0	8 65
4 0	8 8	3 0	7 0	7 57	10 6	5 0	9 45
5 0	9 65	4 0	9 05	8 35	11 1	6 0	9 85
5 9	10 0	7 25	10 8	8 9	11 2	6 85	10 15
7 35	10 75	8 25	11 15	9 6	11 4	7 8	10 7
8 0	10 95	9 43	11 3	10 4	11 6	8 65	11 06
8 97	11 2						*(10 9+ 0 18)
10 0	11 5						
11 3	11 6						
11 7–9	11 75						
	*(11 6+ 0 18)						

* Colorimetric values with protein error correction

[*Note added February* 12 —An inflection on the acid side of the curve was reported by Booth (1930) These results, however, are not comparable with ours for several reasons The solutions contained a much higher concentration, namely 0 03 equivalents per litre, of ammonium sulphate, which obviously affected the blank corrections, and probably also the course of the curve Further, the acid titrations were done with the quinhydrone which in physiological protein solutions has proved uncertain in its action owing to its oxidation potential The alkaline titrations were done with the glass electrode, and in the presence of formaldehyde Both series start from about p_H 7 0 instead of the isoelectric point, p_H 4 7]

It can be shown by the equivalent weights of the fission products that a great reserve of carboxyl and other acidic groups exists both in gelatin and in albumin In gelatin, however, a definite proportion of these has been set free for combination with alkali, but this action nearly ceases after 8 c c of alkali have been added Albumin, on the other hand, especially if in the form of metaprotein, continues to combine with alkali up to at least 12 c c (corrected for alkali uncombined)

The curve of combination with 0 1 per cent albumin resembles the lower curves of 1 0 per cent solutions

Table IV —Stock Solutions A and D dialysed as above

0 1 per cent solutions		Alkali titrations 25° C	
A		D	
c c 0 1N alkali to 1 g	p_H	c c 0 1N alkali to 1 g	p_H
1 0	5 4	2 0	5 6
2 0	6 0	3 0	6 4
3 0	6 75	4 0	7 4
4 0	7 4	5 0	7 9
5 0	7 6	6 67	8 4
7 0	8 0	7 0	8 45
10 0	8 15	8 0	8 65
12 0	8 2	9 0	8 75
20 0	8 5	10 67	9 0
30 0	8 8	12 0	9 05
		13 33	9 3
		14 0	9 35
		16 0	9 65

Denaturation —The alteration in the nature of albumin by treatment with acids or alkalies is widely regarded as due to hydrolysis (Lepeschkin, 1922).

The change in the combination curves due to various forms of denaturation was investigated by Hendrix and Wilson (1928) up to p_H 7 9 by means of the quinhydrone electrode Those for metaprotein were stated to be parallel to those for fresh albumin, as we have found Denaturation by heat coagulation may be of quite a different type, owing to a dehydration of the whole molecule, T B Robertson (1924) The effect of heat was investigated by Hendrix and Wilson (*loc cit*), the albumin being coagulated by heating it for 30 minutes in an autoclave, and afterwards drying it at 60° The results fig 1, show that there was nearly complete loss of buffering power above p_H 6, but a return to normal values after nearly 3 c c of alkali had been added

A sample was denatured by precipitation with alcohol and titrated with alkali The results are given in Table V and plotted in fig 1 The points as seen on the curve are intermediate between the lowest and the highest but between p_H 6 and 9 are rather high, somewhat resembling those of the heat coagulated sample. The whole curve resembles most closely that for good commercial dried albumin

Table V —Comparison of albumin denatured by alcohol A with good dried commercial sample B Additions of 0 1N acid or alkali to 1 g of albumin in 1 per cent solution

Alcohol denatured 1 per cent solutions				Dried albumin (commercial sample) 25° C			
Alkali				Acid		Alkali	
c c 0 1N alkali	p_H	c c 0 1N alkali	p_H	c c 0 1N acid	p_H	c c 0 1N alkali	p_H
1 40	7 6	1 35	6 4	0 65	4 5	0 83	5 45
2 60	8 25	1 96	7 15	1 52	4 2	1 66	6 1
4 60	9 0	3 06	8 5	2 50	3 9	2 42	7 6
5 60	9 2	4 60	9 05	3 45	3 75	3 17	8 2
6 60	9 4	6 1	9 4	5 34	3 35	3 92	8 5
7 60	9 65	7 73	9 7	6 74	3 05	4 68	8 8
8 60	9 9	9 2	10 0	7 60	2 7	6 19	9 2
9 4	10 15			8 0	2 4	7 70	9 6
10 4	10 25					9 2	9 9
12 1	10 6					10 4	10 3
13 9	10 75					11 8	10 6
15 64	10 9						

Buffering Power and Equivalent Weight

A greater length of the straight part or branch of the curve corresponds to a greater number of reacting groups per 1 gr of substance, and to a smaller equivalent weight. The equivalent weight of gelatin as base is less than that as acid, the ratio being about 0·9, Prideaux (1924), *loc. cit.* From inspection of the curve it would seem that a similar relation holds for albumin, although the failure of inflections makes it impossible to determine titration equivalents. The buffer capacity, however, as already shown, gives information regarding the equivalent weights due to those groups which determine p_H in any chosen part of the curve, preferably the middle of a straight branch.

In the case of gelatin, the buffering power B is uniform from p_H 3·0 to 5·0, $\Delta V/\Delta p_H = 3·9$, $\Delta C/\Delta p_H = B = 3·9 \times 10^{-4}$, equivalent $= 0·576/B = 1470$. The corresponding value for albumin, $B = 3·5 \times 10^{-4}$, is not constant over the range but is represented by the chord of an arc, since the values of B are already beginning to vary from p_H 4·0 as is shown by the series:—

p_H range =	2·6–4·4	3·0–5·0	3·0–4·0	4·0–4·7	4·7–6·0	6·0–8·0
10^4B =	5·1	3·5	4·7	2·6	1·7	0·75

The equivalent should be defined by B on a straight part of the curve which in this case does not include pI but lies between 2·6 and 4·4. Adopting a common convention by which all constants are expressed as acid constants, albumin chloride* behaves here as an acid having $pk_A = 3·5$ and an equivalent, calculated as above, equal to 1130. If regarded as a base, albumin would have the same equivalent and $pk_B = 10·7$. The buffering power then rapidly diminishes, reaching a minimum of about 0·75 ($= 10^4B$) in the middle of the inflection. Among all the dissociating groups of albumin (and also gelatin) there are few or none having pK_A or pK_B in the neighbourhood of 7.

In the alkaline range, there is a rectilinear branch from about p_H 10·3 to 11·3 or 11·0 to 11·6, the corresponding values of 10^4B are 4·0 or 4·3 and the mean equivalent as acid is *1390*. The value of 10^4B for gelatin at p_H 10 to 11 is about 2·0 and the corresponding equivalent weight is *2880* or about twice that of albumin.

The ratios of the basic to the acidic equivalent weights of gelatin, calculated in this way, are much lower than those obtained by other methods, and, as already stated, only represent the behaviour in the buffered parts of the curves.

For gelatin—

$$\frac{\text{Equivalent as base about } p_H \ 4·5}{\text{Equivalent as acid about } p_H \ 10·5} = \frac{1470}{2880} = 0·51$$

---

* The apparent acid constant has this meaning also in the former paper (1931, p. 225).

For albumin—

$$\frac{\text{Equivalent as base about } p_H \ 3 \ 5}{\text{Equivalent as acid about } p_H \ 10\cdot 8 \ (\text{or } 11\cdot 3)} = \frac{1130}{1390} = 0\cdot 812$$

Albumin has the better buffering power in both branches and the basic and acidic values are not so unequal as those of gelatin. The buffering power of aged, alkali treated, or 0·1 per cent. albumin is even greater. It is estimated that in the alkali range 10^4B is 5·7 and the apparent equivalent is nearly 1000

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Uteroverdin, the Green Pigment of the Dog's Placenta.

By R. Lemberg and J Barcroft, F R S

(Received January 18, 1932.)

(From the Biochemical and Physiological Laboratories, Cambridge.)

[Plate 18]

In investigations made by one of us (in collaboration with P. Rothschild (1931)) on the blood content of the canine uterus, difficulties arose in the hæmoglobin determinations owing to the presence of a green pigment in that organ The other author (Lemberg, 1931) had at the time just investigated the green pigment contained in the egg-shells of many birds and had isolated its methyl-ester in crystalline form, he at once realised that the pigment mentioned above was of the same nature and furnished a profitable field for investigation We must here thank Professor Keilin for his valuable help in the preliminary stages of the research.

There is considerable literature on the subject in papers of Medicine and Anatomy, but so far as modern physiological and biochemical publications are concerned, it has been forgotten, there being no recent chemical work on the subject

The pigment was discovered by Breschet (1830) This work also contains a chemical investigation by Barruel, who extracted the pigment from the placenta with alcohol and found a close resemblance to " biliverdin " from ox bile Since then only investigations of minor chemical importance have been made Meckel (1852) believed it to be derived from oxy-hæmoglobin by the action of oxygen and gave it the name hæmatochlorin Meckel, on the alleged authority of Barruel, alluded to the " ether solubility " of the pigment, but we have been unable to discover any mention of this important property in Barruel's work

O. Nasse (1863) found a similar pigment in the epithelium surrounding the yolk-sac of the shrew and reported its extractibility with ether

Bischoff (1845) found, besides the green pigment, water-soluble crystals in the green mass filling the borders of the zonary placenta and is misquoted by Preyer (1871), who stated that Bischoff found the green pigment itself as crystals.

Preyer was the first to examine the spectrum which shows no bands, but absorption of light in the red. He denied its identity with biliverdin on account of its slight solubility in water and described hæmatoidin crystals as being associated with it

Etti (1871) extracted the pigment with alcohol-sulphuric acid and first considered it to be biliverdin associated with "biliprasin," but, yielding to the criticism of Maly, subsequently regarded it as biliverdin only. He extracted with ether a substance similar to "hydrobilirubin," which we can now infer to have been due to the contamination of his ether with peroxide which oxidises the uteroverdin Thudichum (1886) assumed a near relationship between the pigment of the dog's placenta and the green pigment of the egg-shells of birds which he (1879) had found to be different from biliverdin both in its chemical character and its physiological formation. He did not, however, investigate the former

Since 1871 no chemical investigations have been carried out This is all the more strange because the pigment has attracted the attention of numerous embryologists, Heinricius (1889), Lieberkühn* (1889), Strahl (1890), Duval (1893), Tafani (1886), Bonnet (1903) and others. Of particular interest are those of Lieberkühn and of Bonnet, as is pointed out by Schick (1921), who discusses the formation of bilirubin from the hæmoglobin of maternal human blood as a cause of *icterus neonatorum*

These authors all agree that the pigment is derived from the hæmoglobin of maternal blood extravasated from hæmorrhages under the chorion These hæmorrhages are most marked just outside the area in which the barrel-shaped ovum is firmly attached to the uterine wall, and in the area in which it is loosely attached Thus two green rings are formed They begin to be formed about the twenty-first day of pregnancy and increase till each band is about 1 cm in breadth, forming together about two-fifths of the whole placenta The green pigment is, however, not confined to the maternal tissue, but is found with other products of blood degeneration in the cells of the ectoderm fœtal villi, but no author suggests that the pigment so found is formed from the fœtal hæmoglobin It is not clear whether the pigment is formed in the ectoderm cells or is only taken up by these phagocytic cells after its extracellular formation in the extravasate

The literature contains no evidence of an extracellular enzyme which converts hæmoglobin into "uteroverdin" (as we will call this pigment†), nor is it proven that the transformation takes place within the bodies of the chorion cells, Stahl and Bonnet expressing opposite views on this subject We have been no more successful than others (Wagner, 1921) in attempts to prove the presence of an extra-cellular enzyme

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* Posthumous paper published by Strahl.

† Mouchet (1928) found apparently enzymic formation of a green pigment from hæmoglobin in the lumen of the intestinal tube of *Gnathia*

Naturally, the ultimate point of interest is whether the iron set free from extravasated hæmoglobin is a provision for the nutrition of the fœtus The only contribution we can make is the statement that we isolated from each of three cases pigment equivalent to about 2·75 grams of hæmoglobin. We consider that the uterus contained uteroverdin corresponding to at least 3–4 grams of hæmoglobin The combined weight of the fœtuses was 528 grams, containing perhaps 40 grams of blood and 4–5 grams of hæmoglobin The uterine pigment corresponded then to little less hæmoglobin than was contained in the embryos

Green pigments are found in such prominence only in the dog's uterus and in the egg-shells of birds, in less degree they occur in the uterus of other species (Marshall, 1922, Recklinghausen, 1883) The green pigment of the meconium is perhaps of the same nature as uteroverdin and will be investigated In other cases hæmotoidin is mentioned

Nomenclature

We have termed the pigment "uteroverdin" Meckel's name—hæmatochlorin—seemed unsuitable because the term "chlorin" is now associated with a definite class of chlorophyll derivatives

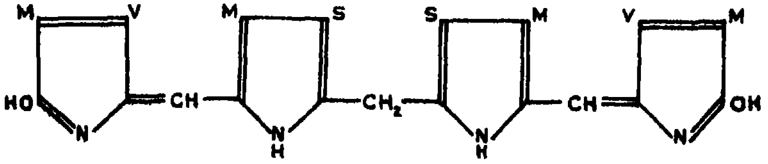
The close relation of oocyan described by Lemberg (1931) suggested the inclusion of "cyan" as part of the name, but the colour in the placenta is definitely green

Chemical Structure, etc

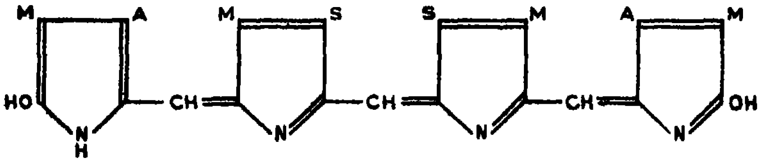
The object of the chemical investigation was to show the relation between the green pigment and bilirubin The name "biliverdin" has been much abused, different pigments, occurring naturally or obtained by the most diverse methods, so long as they were soluble in alcohol and showed the Gmelin test, have been alluded to as "biliverdin" The formula of the biliverdin obtained by the oxidation of the sodium salt of bilirubin by atmospheric oxygen is usually given as $C_{33}H_{36}N_4O_8$, but this is not a single substance, as is established by the investigation of the products of its methylation (Lemberg, 1931) The greater part of its methyl-ester is insoluble in ether No "biliverdin" has been obtained in a crystalline state Oocyan, sometimes referred to as biliverdin, has been shown to be quite different from the substance mentioned above It is characterised by its ether solubility, its comparatively strong basicity, the slight solubility of its hydrochloride and the blue colour of the ether solution. Uteroverdin is a second example of this class of substances. As already stated in the paper on oocyan (Lemberg, 1931), the properties of uteroverdin are extremely similar to those of oocyan—a fact which made isolation much easier, as we could predict the whole behaviour of the substance.

At first only the placentas of one uterus were at our disposal; isolation had to be as quantitative as possible. On the other hand, the risk of alteration had to be avoided. It was possible to do both simultaneously. We obtained from the uterus enough crystalline uteroverdin-methyl-ester to make a complete analysis in duplicate. According to this analysis and some crystallographic facts, oocyan and uteroverdin are different. The analysis of the uteroverdin-methyl-ester showed its composition to be $C_{35}H_{38-42}N_4O_6$, the methoxyl determination giving two methoxyl groups. According to this, the formula of uteroverdin is $C_{33}H_{34-38}N_4O_6$ and a formula with only three pyrrol nuclei as assumed for oocyan does not come into consideration for uteroverdin. We are probably dealing with a dimethyl-ester of dehydrobilirubin or dehydromesobilirubin.

Bilirubin being, according to H. Fischer's recent investigations (1931):



a formula like



$$M = CH_3, \quad A = C_2H_5, \quad V = C_2H_3, \quad S = C_2H_4 . CO_2H$$

may be regarded provisionally as that of uteroverdin.

The values for C, H and N, but not the methoxyl content of oocyan-ester, agree also with the formula $C_{37}H_{42}N_4O_8$, that is to say, with that of a trimethyl-ester of a substance which differs from uteroverdin only in possessing one more carboxyl group. Both uteroverdin and oocyan give an excellent Gmelin test in chloroform solutions with concentrated nitric acid. Recently Hans

Fischer and Adler (1931) have stated that their synthetic tripyrrenes, though similar to oocyan in some respects, do not show absorption of light in the red nor give the Gmelin test. As different isomerides of tripyrrenes are possible, with different positions of the double bonds, these facts do not disprove the assumption of a tripyrrene system for oocyan The properties of uteroverdin and oocyan are, however, so similar that the assumption that the latter belongs to a class with three, and the former to one with four pyrrol nuclei is very improbable Both pigments show almost the same colour and absorption curve in neutral and acid solutions, their solutions in ether showing the bluest colour. Both are extracted from their ether solutions by 0·1 normal HCl and the hydrochlorides are with difficulty soluble in water The methyl-ester of each crystallises from methyl alcohol, and when the two methyl-esters are mixed they give no distinct depression of the melting point Further investigation of oocyan by one of us (R L) is in progress.

The fact that uteroverdin corresponds to "biliverdin" in its absorption of light and in its behaviour to the Gmelin test is of chemical interest It means that the two oxygen atoms, added in the oxidation which converts bilirubin into biliverdin, are probably taken up by the unsaturated side chains of bilirubin Mesobiliverdin should contain only six oxygen atoms. The green substances which may be prepared from bilirubin and mesobilirubin will be investigated From this point of view the green pigment of bird's bile is interesting It is perhaps a definite product of the liver cell, not merely being formed by the oxidation of bilirubin by atmospheric oxygen as in the case of the biliverdin of ox bile

Preparation and Properties

The zonary placenta of the dog, which is approaching full term, is easily dissected from the uterus and may be taken away but little contaminated with maternal hæmoglobin. When the uterus is opened, and the fœtus gently pulled, the natural cleavage is between the pigmented part and the rest of the uterus, a little help with scissors bringing the pigmented placenta cleanly away attached by the cord to the fœtus The cord is then cut We have observed the pigment in the placentas attached both to dead and living fœtuses

The placentas are placed in a dish with distilled water, the veins are opened by fine scissors and the blood is allowed to pass into the water. The resulting solution of blood, on standing, deposits a little green pigment from which the hæmoglobin solution is poured off From the bled and washed placentas the broad, intensely green margins are now cut off. Sometimes the pigment is present only in the margins, but on occasions large pockets of pigment occur in the middle of the placentas, these are excised and added to the margins In the first case a ring of tissue, 1 inch broad, remains, but in the outer layer small green grains are still to be found. This is therefore cut off and worked up separately The remaining zone of flesh is free from pigment and is thrown away The collected margins and pouches of pigment are now cut open, the green granular mass scraped out, and washed out with water The washings are filtered off from the membranes through muslin, and the procedure of scraping out and washing repeated until the water no longer brings out any appreciable amount of pigment (A) The remaining mass, however, still contains much pigment, and is therefore extracted, together with the muslin, with 10 per cent HCl in methyl alcohol (B) The outer layer cut off from the placenta is washed with water, the washings are filtered through muslin, the solution, which still contains hæmoglobin, is poured off from the pigment which settles down The pigment precipitates are extracted with HCl in methyl alcohol in the same way as the particles of membranes and tissue which remain on the muslin (C) The aqueous suspension, which contains the greater part of the pigment, is centrifuged. The green mass which is thrown down is brought into solution in about 10 per cent HCl in methyl alcohol by grinding in a mortar (D) After standing for 24 hours it is filtered and the extraction repeated after further grinding (E) In the investigation of the first placenta the extraction was at first carried out with methyl alcohol alone instead of with HCl in methyl alcohol, in order to avoid a possible alteration, a considerable part of the pigment goes into solution, and may be brought into ether in the manner described below, showing the properties of unesterified uteroverdin The aqueous solution, from which the greater part of the pigment has been removed by centrifuging, still contains considerable amounts in very fine suspension, besides some hæmoglobin and hæmatin. It is treated in the following way it is acidified with HCl up to a content of about 5 per cent, and then the hæmatin extracted with a mixture of ether and glacial acetic acid. Uteroverdin remains in the HCl solution. The flocks of protein and hæmatin which come down in the boundary layer still contain green pigment. They are collected separately, dissolved in methyl alcohol (ethyl alcohol is avoided, as a partial esterification occurs) and again brought into a mixture of glacial acetic acid, ether and dilute HCl. This process is repeated until the flocks yield no more green pigment even in hot methyl alcohol. All the flocks which separate out, and the residues filtered off in this course of the preparation, must be carefully worked up in this way, as they often include considerable amounts of pigment Emulsions are broken by the addition of some methyl alcohol to the ethereal solution. One obtains finally a blue-green solution of uteroverdin in HCl and a brown solution of hæmatin in ether The latter is once more extracted with 1 per cent. HCl, and all the hydrochloric acid solutions are filtered without being allowed to stand for long The HCl solution is once more shaken out with a little ether, then the acid is almost neutralised with sodium carbonate, and the pigment is brought into ether by adding concentrated sodium acetate solution and repeatedly shaking out with ether The last fraction is brought into ether by allowing the aqueous solution to stand with ether after saturation with sodium acetate The ethereal solution is then thoroughly washed with water, to which at first some sodium acetate is added

The methyl alcoholic solutions previously obtained (B, C and E) must first be concentrated by vacuum distillation The frothing, which is produced by protein and cholesterol separating out, prevents further evaporation, but if it is allowed to stand for a little time and filtered it can be further concentrated to 100 c c The solution richest in pigment (D) is concentrated by itself to 100 c.c , the united methyl alcoholic solutions are neutralised with sodium carbonate till they give a weak reaction with Congo red and then added to a mixture of ether and dilute sodium acetate solution The uteroverdin is brought into ether in the manner described above The united blue-green ethereal solutions are thoroughly washed with water The uteroverdin is now extracted with an abundance of 1 per cent. aqueous HCl Here one must work quickly, as the uteroverdin hydrochloride is only slightly soluble in HCl At least an equal volume of HCl is therefore added immediately to the ethereal solution After two or three extractions the ether remains brown and contains only hæmatin The hydrochloric acid solution is shaken immediately with a little ether in order to remove the last traces of hæmatin. After standing for a short time the hydrochloride of uteroverdin separates out from the blue-green solution in fine, shining platelets The precipitate is filtered off and dried in an evacuated desiccator The filtrate is partially neutralised with sodium carbonate, mixed with sodium acetate and extracted with ether , the ethereal solution is thoroughly washed and evaporated down in a stream of nitrogen at low pressure It is dried *in vacuo* at 50° and the residue, together with the dried precipitate which was filtered off, dissolved in methyl alcohol containing HCl. It is esterified by standing for 12 hours in 15 per cent HCl in absolute methyl alcohol, taken down to dryness *in vacuo*, the residue digested with ether and dissolved in a little methyl alcohol. This solution is introduced into a mixture of ether and dilute sodium acetate solution to get the free ester The flocks which at first separate out at the boundary layer go into the ethereal solution on further shaking out with ether This solution is repeatedly washed with 2 per cent sodium carbonate solution which takes out a little unesterified uteroverdin and then immediately with much water, filtered through a dry filter, dried with sodium sulphate, and evaporated down *in vacuo* in a stream of nitrogen The ether must be absolutely free from peroxide, as otherwise the uteroverdin is rapidly oxidised to a blue "bilicyanin"-like, or a red-violet pigment which is soluble in petroleum ether The residue from the ethereal solution is digested with petroleum ether and a little ether, taken up in hot absolute methyl alcohol, and the solution is then concentrated On standing under nitrogen the methyl-ester of uteroverdin separates out in flat platelets, which are apparently completely similar to those of the ester of oocyan. (Dark green platelets with a violet surface colour, sometimes also coarse four-sided prisms, some with straight ends, some with oblique or wedge-shaped ends, fig 1, Plate 18) The solubility in methyl alcohol decreases on repeated recrystallisation 45 mg of crystalline uteroverdin ester were isolated from the first uterus, and 300 mg were obtained from three others In spite of the similarity in form the crystals are not identical with those of oocyan-methyl-ester We have to thank Dr Bernal for the following comparison of the uteroverdin and oocyan-methyl-esters —

	Uteroverdin-methyl-ester	Oocyan-methyl-ester
In unpolarised light	Bright green—dark green-blue.	Bright blue—dark green-blue
In polarised light—		
Plane of polarisation		
(*a*) Parallel to long axis of platelets	Green	Dark blue
(*b*) Perpendicular to long axis	Blue	White
Between crossed Nicols' prisms		
(*a*) In rectangular position	Approximate extinction, dark red	Blue (changes on turning the crystal to red, green, green-yellow)
(*b*) 45° position	Bright green.	—

The melting point of uteroverdin is 199° C. (corrected), with decomposition. On rapid heating it is found to be higher. A mixture with oocyan ester melts at the same temperature or some degrees higher. Oocyan has a higher melting point—233–234° C

The micro-analyses were carried out in the Kaiser Wilhelm Inst. f mediz. Forschung at Heidelberg by Dr Roth. The substance was dried in high vacuum at the temperature of boiling alcohol

2·753 mg substance—6 875 mg. CO_2, 1·60 mg H_2O
3 579 mg substance—8 89 mg CO_2, 2·04 mg H_2O
3 01 mg substance—0·237 c c N_2 (at 751 mm and 20° C)

Methoxyl—

3 824 mg substance—2 63 mg AgI
2 155 mg substance—1 47 mg AgI

Calculated for—

$C_{35}H_{38}N_4O_6$ C = 68 82, H = 6 28, N = 9·18, 2 OCH_3 = 10·16
$C_{35}H_{42}N_4O_6$ C = 68 37, H = 6 89, N = 9 12, 2 OCH_3 = 10·10.

Found—

$$\left.\begin{matrix} C = 68\ 10 \\ 68\ 08 \end{matrix}\right\} \left.\begin{matrix} H = 6\ 50 \\ 6{\cdot}42 \end{matrix}\right\} N = 9{\cdot}05,\ OCH_3 = \left.\begin{matrix} 9\ 09 \\ 9\ 03 \end{matrix}\right\}$$

Uteroverdin ester gives a definite Gmelin reaction, as does oocyan ester. This is best carried out by adding conc nitric acid to its solution in chloroform But even when using dilute sulphuric acid and nitrite the blue stage can be observed if only very little nitrite is taken Both substances behave in a completely analogous way on the appearance of the blue colour a band appears at 630–600 mμ in the case of uteroverdin, and 620–590 mμ with oocyan, besides which a weaker band can be seen at about 550 mμ The solution becomes blue-violet and then red-violet In the first stage the second band becomes stronger, the first moving towards the red Afterwards the urobilin band (495 mμ) appears.

The absorption curves, fig 2, Plate 18, of uteroverdin are similar to those of oocyan, both in acid and in neutral solution Free uteroverdin, like free oocyan in ether, is a greener blue than the corresponding ester.

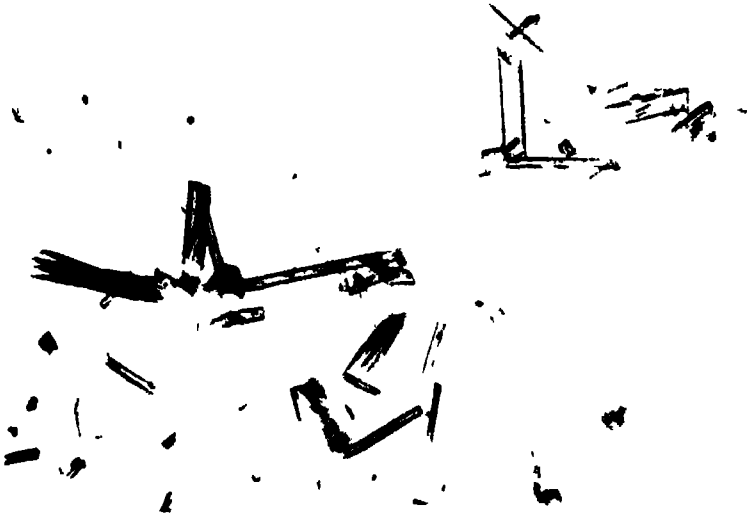


Fig. 1 Crystals of the methyl ester of uteroverdin

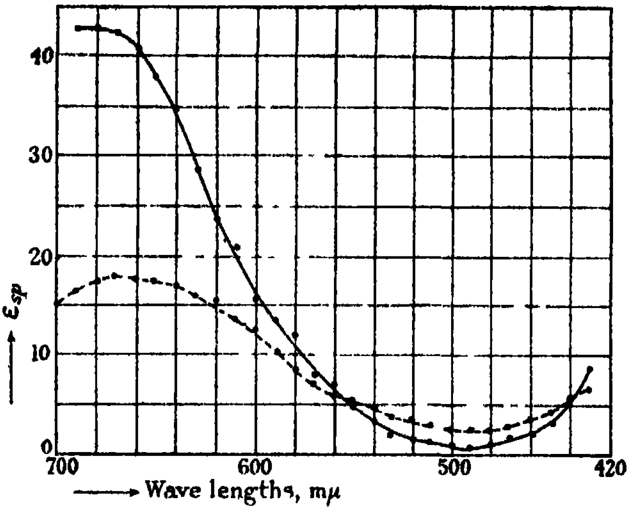


FIG. 2

Photometric absorption curves of uteroverdin —— in $CH_3OH + 5$ per cent HCl. ... in neutral CH_3OH

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The Trypanocidal Action of some Derivatives of Anil and Styryl Quinolines.—II *

By C H Browning, F R S, J B Cohen, F R S, K E Cooper, and R. Gulbransen

(Received January 30, 1932)

(From the Medical School, Leeds, the Pathological Department of the University and the Western Infirmary, Glasgow)

The substances whose actions are described here are analogues of compounds already investigated (Browning, Cohen, Ellingworth and Gulbransen) The method used in testing for trypanocidal action was that previously detailed Mice 24 hours after inoculation with *T brucei*, when scanty parasites were present in the blood, received a subcutaneous injection of a solution of the drug. The results are shown in the table Animals infected with the strain of parasites employed were cured by the active styryl quinoline compounds Nos 8 and 90, in the doses previously stated

Anil quinolines—Nos 352, 353, 350, 351, 358 and 360, like their analogue 2(*p*-dimethylamino anil)-6 acetylamino quinoline methochloride (No. 59) already examined, manifested only a slight to marked trypanocidal action when doses approaching the maximum tolerated by the animals were used.

Styryl quinolines.—With Nos. 359, 361 and 412—analogues of 2(*p*-amino styryl)-6 acetylamino quinoline methochloride (No 8)—cures were effected

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* The work reported in this communication was done with the support of the Medical Research Council

in every case, but on testing a series of doses the range of action of these substances appeared to be less than that of No 8 2(*p*-lactylamino styryl)-6 amino quinoline methochloride (No. 391) had practically the same range of action as No 8 On acetylation of the amino group in the quinoline nucleus of No 391 (No 397) the trypanocidal properties did not appear to be markedly altered, the latter substance tended to produce convulsive effects, a character exhibited by other compounds whose amino groups in both nuclei have been acylated, *e g*, No 88 The convulsant action was marked in No 417, the trypanocidal property of the latter was well developed No 398, the acetyl lactylamino analogue of the quaternary compound of 2(*p*-amino styryl) acetylamino benzthiazol (No 332) and No 392, the similar analogue of 2(*p*-acetylamino styryl) amino benzthiazole (No 368), had trypanocidal activity of nearly the same order as the compounds with the free amino groups

Experimental

Acetyl lactylamino quinaldine—*p*-amino quinaldine (5 g) were dissolved in chloroform and a solution in chloroform of acetyl lactyl chloride (4·8 g) prepared by the method of Anschutz and Bertram (1904) slowly added Heat is evolved and the reaction is completed by heating on the water bath for half-an-hour On distilling off most of the chloroform the hydrochloride of acetyl lactylamino quinaldine crystallises with chloroform of crystallisation, yield is 11 4 g It was dissolved in water, made just alkaline with ammonia, extracted with chloroform and dried with sodium sulphate On removing the solvent, a sticky mass remained which on rubbing with a little ether crystallised After recrystallisation from benzene, it separated in colourless plates, m p 141–142°

Acetyl lactylamino quinaldine methosulphate - The base (4 g) was dissolved in benzene and heated on the steam bath with dimethyl sulphate (1·5 c.c.) for 2 hours A pale yellow solid separated which was filtered and washed with benzene It was recrystallised from alcohol, yield 3 8 g Found, N, 7·10 per cent, $C_{17}H_{22}O_7N_2S$ requires N, 7 02 per cent The methochloride is obtained by adding a saturated salt solution to a concentrated solution of the methosulphate.

6-*Lactylamino quinaldine*—The above acetyl derivative (5 g) was shaken with 80 c c. normal sodium hydroxide solution On standing, crystals of the hydrate separated and were filtered, washed and dried It loses water at 100°, solidifies and melts at 192° (yield 3·6 g)

6-Lactylamino quinaldine methosulphate.—Was prepared by heating the base and methyl sulphate in nitrobenzene on the steam bath It crystallised from alcohol in yellow needles Found, N, 8·00 per cent , $C_{15}H_{20}O_6N_2S$ requires N, 7·87 per cent

Acetyl lactylamino benzaldehyde—Freshly prepared aminobenzaldehyde (4 0 g.) were dissolved in chloroform and pyridine (6 c c) added After cooling in water, acetyl lactyl chloride (5 5 g) in 10 c c of chloroform were added and the mixture left for some time The reaction was completed on the steam bath The chloroform was distilled and the last traces removed in a vacuum desiccator. The solution was extracted with water several times to remove pyridine, and finally with the addition of a little ammonia The solid product was crystallised from benzene several times , m p 114–115° It forms colourless crystals Found, N, 6 4 per cent , $C_{12}H_{13}O_4N$ requires N, 6 per cent

Acetyl lactylamino benzthiazole—Amino 2-methylbenzthiazole (4 3 g) were dissolved in chloroform and acetyl lactyl chloride (5 g) added The mixture was boiled for 2 hours, filtered and the chloroform distilled The product, which is a mixture of the base and hydrochloride, was made just alkaline with ammonia, filtered, washed and dried. Recrystallised from benzene it forms colourless crystals , m p 153–155°

Acetyl lactylamino 2-methyl benzthiazole methosulphate was prepared by dissolving the base (1 5 g) in benzene, adding neutral methyl sulphate (0·7 g) and refluxing on the steam bath for 3 hours The methosulphate crystallises on cooling and is washed with benzene and dried

Styryl and anil derivatives of acetyl lactylamino and lactylamino-quinaldine methochloride are obtained by heating on the steam bath theoretical quantities of the quinaldine and *p*-amino benzaldehyde or nitroso dimethylaniline with a few drops of piperidine for 1–2 hours On cooling, the anil or styryl crystallised and were recrystallised from alcohol or dilute alcohol (styryls, 359 and 361, anils, 358 and 360)

Styryl derivatives of acetyl lactylamino benzthiazole and of acetyl lactylamino benzaldehyde—No piperidine is used in these condensations In the preparation of 2(*p*-amino styryl) acetyl lactylamino benzthiazole methochloride, the crude methosulphate is used for the condensation. As the product does not crystallise, the alcohol is removed, the residue boiled with water and charcoal filtered and precipitated as the iodide, which in turn is converted into the chloride by boiling with silver chloride in methyl alcohol. After crystallisation from methyl alcohol, it forms long violet needles Found, Cl, 8 per cent , $C_{21}H_{22}N_3O_3SCl$ requires Cl, 8·2 per cent. (398). In the case of 2(*p*-acetyl lactylamino styryl) amino benzthiazole methochloride, a similar procedure was followed as above and the sulphate converted to the chloride by the addition of saturated salt solution. It was finally crystallised from alcohol It formed dark red plates Found, Cl, 8·01 per cent , $C_{21}H_{22}O_3N_3SCl$ requires Cl, 8 2 per cent (392)

2(*p-acetyl lactylamino styryl*)-6 *amino quinoline methochloride* is carried out in the usual way , but on condensation the acetyl group is hydrolysed, yielding the lactylamino styryl derivative Found, Cl, 9 25 per cent , $C_{21}H_{22}O_2N_3Cl$ requires Cl, 9·3 per cent. (391) Also in the condensation of acetyl lactylamino benzaldehyde with acetylamino quinaldine methiodide, hydrolysis of the acetyl group in the former occurs giving 2(*p*-lactylamino styryl)-6 acetylamino quinoline methiodide Found, I, 24·4 per cent , $C_{23}H_{24}O_3N_3I$ requires I, 24·6 per cent (397)

6-*Diacetyl glycerylamino quinaldine methiodide*.—Freshly prepared glyceric acid (50 g) were added in small quantities to acetyl chloride (100 c c) and cooled in ice water if the reaction is too vigorous or warmed gently on the steam bath if the syrup fails to dissolve The mixture is left at room temperature overnight and then refluxed on the steam bath for half-an-hour. After cooling, 70 c c of thionyl chloride are added and the mixture warmed cautiously and finally refluxed for 2 hours on the steam bath The excess of thionyl and acetyl chlorides are then removed by distilling *in vacuo* at 35° The residual syrup is distilled rapidly at 15 mm or less until the boiling point reaches 100°. Yield, 7 g., boiling at 75–85° at 15 mm The diacetylglyceryl chloride dissolved in an equal volume of chloroform is added to aminoquinaldine (10 g) in chloroform. Heat is evolved and when the reaction has moderated the reaction is completed by refluxing on the steam bath for 2 hours The precipitated aminoquinaldine hydrochloride is filtered and the chloroform solution evaporated. The gummy residue is washed with dilute ammonia and water, extracted with chloroform, dried with anhydrous sodium sulphate and the chloroform removed The product which did not crystallise was converted directly into the methiodide by heating with excess of methyl iodide for 3 hours on the steam bath.

The crystalline methiodide was recrystallised from alcohol and forms colourless needles , m p 217–219° (decomp) Found, I, 27·1 per cent ; $C_{18}H_{21}O_5N_2I$ requires I, 26·9 per cent. In the preparation of 2(*p-acetylamino styryl*)-6 *glycerylamino quinoline methiodide* and 2(*p-amino styryl*)-6 *glycerylamino quinoline methiodide*, 6 diacetyl glycerylamino quinaldine methiodide was used, but in process of condensation the acetyl groups were removed

The condensations were effected in methyl alcohol with the addition of piperidine In the first case found, I, 24·0 per cent , $C_{23}H_{24}O_4N_3I$ requires I,

Table

No	Substance	Dose	Result
	Anils		
352	2(*p*-dimethylamino anil) 6 undecylenylamino quinoline methochloride	1 1000	Slight
353	2(*p*-dimethylamino anil) 6 laurylamino quinoline methochloride	1 1000	Slight
350	2(*p* dimethylamino anil) 6 caprylylamino quinoline methochloride	1 500	Slight
351	2(*p* dimethylamino anil) 6 pelargonylamino quinoline methochloride	1 500	Slight
358	2(*p*-dimethylamino anil)-6 acetyl lactylamino quinoline methochloride	1 500	Slight
360	2(*p*-dimethylamino anil) 6 lactylamino quinoline methochloride	1 100	Marked
	Styryls		
359	2(*p* amino styryl) 6 acetyl lactylamino quinoline methochloride	1 1000– 1 7500	Cure
		1 10000	Marked
361	2(*p* amino styryl) 6 lactylamino quinoline methochloride	1 1000	Cure
		1 5000	(Cure)
412	2(*p* amino styryl) 6 glycerylamino quinoline methochloride	1 500 1 5000	Cure
		1 10000	Marked
391	2(*p* lactylamino styryl) 6 amino quinoline methochloride	1 1000– 1 15000	Cure
		1 20000	Marked
397	2(*p* lactylamino styryl) 6 acetylamino quinoline methochloride	1 700– 1 7500	Cure
		1 10000	Marked
417	2(*p* acetylamino styryl) 6 glycerylamino quinoline methochloride	1 5000– 1 10000	Cure
		1 15000	Slight
398	2(*p*-amino styryl) acetyl lactylamino benzthiazole methochloride	1 600– 1 1000	Cure
		1 2500	Marked
		1 5000	Slight
392	2(*p*-acetyl lactylamino styryl) amino benzthiazole methochloride	1 750	Cure
		1 5000	(Cure)
		1 10000	Slight

The *doses shown are reckoned per 20 gram body-weight of the mouse* The highest dose shown for each substance is one which approximates to the largest amount borne by uninfected animals without producing obvious toxic effects, *e g* , loss of weight

Note.—The terms used to designate the lesser degrees of trypanocidal action are as follows :—

Trace = Prolongation of life for several days beyond that of the untreated controls

Slight = Disappearance of parasites from the blood for several days to a week

Marked = Absence of parasites from the blood for 10 days or longer

(Cure) indicates that with the doses shown cure was effected only in a proportion of the animals treated

23·8 per cent. In the second case found, I, 24·9, $C_{21}H_{23}O_3N_2I$ requires I, 25·8 per cent. The monoacetylglyceryl derivative requires I, 23 8 per cent The high analytical number in the second compound may be due to incomplete hydrolysis Both compounds were converted into the methochlorides with silver chloride and recrystallised from methyl alcohol (417 and 412)

Caprylyl (350), *pelargonyl* (351), *undecylenyl* (352), *and lauryl* (353) *amino quinaldine derivatives* were prepared from the acid chloride (made by the action of thionyl chloride on the acid) added to an acetic acid solution of aminoquinaldine and heated on the steam bath for 1 hour The product was diluted with water, precipitated with ammonia and crystallised from dilute alcohol The methiodides were obtained by heating the substances in sealed tubes with methyl iodide at 110–120° for several hours The methiodides crystallise from alcohol in pale yellow needles and decompose on heating The condensations proceed normally and the methiodides were converted into the methochlorides in the usual way

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591 157 595 78

Induced Melanism in Lepidoptera.

By A W McKenny Hughes, D I C

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Introduction

In two previous papers (Harrison and Garrett, 1926, and Harrison, 1928) the experimental production of melanic varieties of the moth *Selenia bilunaria*, Esper, was reported, following upon the feeding of the larvæ upon hawthorn leaves which had absorbed small quantities of lead and manganese salts respectively These induced melanic forms were inherited in subsequent generations as recessives on ordinary mendelian lines The production of these melanics was associated by Harrison with the occurrence of melanics in various species of Lepidoptera in industrial areas both in England and Germany where it might be expected that the food plants would become contaminated with metallic fumes

The production of a variation so striking and heritable in what had hitherto appeared to be a fairly stable species seemed to call for further experimental investigation, especially as a natural melanic in the species had been reported (Mansbridge, 1927–28) While it is known that certain violent disturbances of the organism, as by treatment by X-rays, may induce " mutations " which are heritable, it is always difficult to be sure that the species in nature does not contain an occasional recessive gene which escapes observation because of the extreme rarity of the matings which would result in the emergence of the recessive phenotype A fresh series of experiments was therefore initiated at the John Innes Horticultural Institution in order to ascertain if melanic variations could be induced with any regularity by the method indicated by Harrison. In any case it was difficult to understand that manganese should be a casual factor, in view of the invariable presence of manganese in plant tissues and the absence of evidence that the proportion is significantly greater in leaves in industrial areas

Seven females of the Geometrid moth *Selenia bilunaria*, Esper, were caught in the spring of 1928 to start the experiment These females were collected from different areas in Southern and Eastern England, as far as possible away from industrial areas Three females were caught in the New Forest, two at Horseheath in Cambridgeshire, and one at Balsham in Cambridgeshire, and one near Warboys in Huntingdonshire Each family was given a letter, A–C being from the New Forest, D and F from Horseheath, E from Balsham and G from Huntingdonshire. In the course of breeding the strongest family, as judged by general behaviour, from each of the three areas was selected for the experiment and the remainder were discarded. The wild moths were in all respects typical spring brood females of *S bilunaria*, and subsequent generations have all been normal, no signs of melanism having appeared at any time The families have been inbred throughout, and the size of the broods has decreased very considerably as will be seen by comparing broods in the tables Family D died out after the fifth inbred generation and the treated broods in family C after the fourth inbred generation The experiment in this family, however, was restarted from a large control brood and two further generations on lead have now been reared Family G has been inbred for six generations, four of which have received lead and manganese treatment. In each family no treatment took place until the third inbred generation, in order to make sure that there was no latent melanism in the stock

Technique and Method of Treatment

The experiments were carried out in a specially built insectary with glass roof and sides shaded with inside and outside blinds On emergence females and males were selected at random as they synchronised, and were placed in a muslin sleeve to pair Usually a large number of pairings were made, more especially in the later generations, as it was found that many of the pairings failed owing to infertility Eggs were laid on the muslin, which was cut up into convenient pieces to fit into a 3-inch glass-topped tin box in the bottom of which a filter paper had been placed The eggs were all counted as the sleeve was cut up When the resulting larvæ hatched they were transferred to a further 3-inch glass-topped tin box containing the food plant *Cratægus oxyacantha* (hawthorn), as it was found that placing the food plant in boxes in which the eggs were hatching was apt to produce mildew on the muslin and to decrease the hatch If the weather was very hot a little water was dropped on to the filter paper at the bottom of the box containing the eggs. When the larvæ had developed to a sufficient size they were transferred to glass cylinders with a partially perforated zinc top and bottom, and the food plant was placed in glass tubes inside. The insects were allowed to pupate in the cylinders, after which they were transferred to shallow wooden boxes containing earth covered with a layer of silver sand Each pupa was then covered with an ordinary glass tumbler and a small stick was placed inside each glass upon which the emerging insect might expand its wings

Treatment of Food Plant

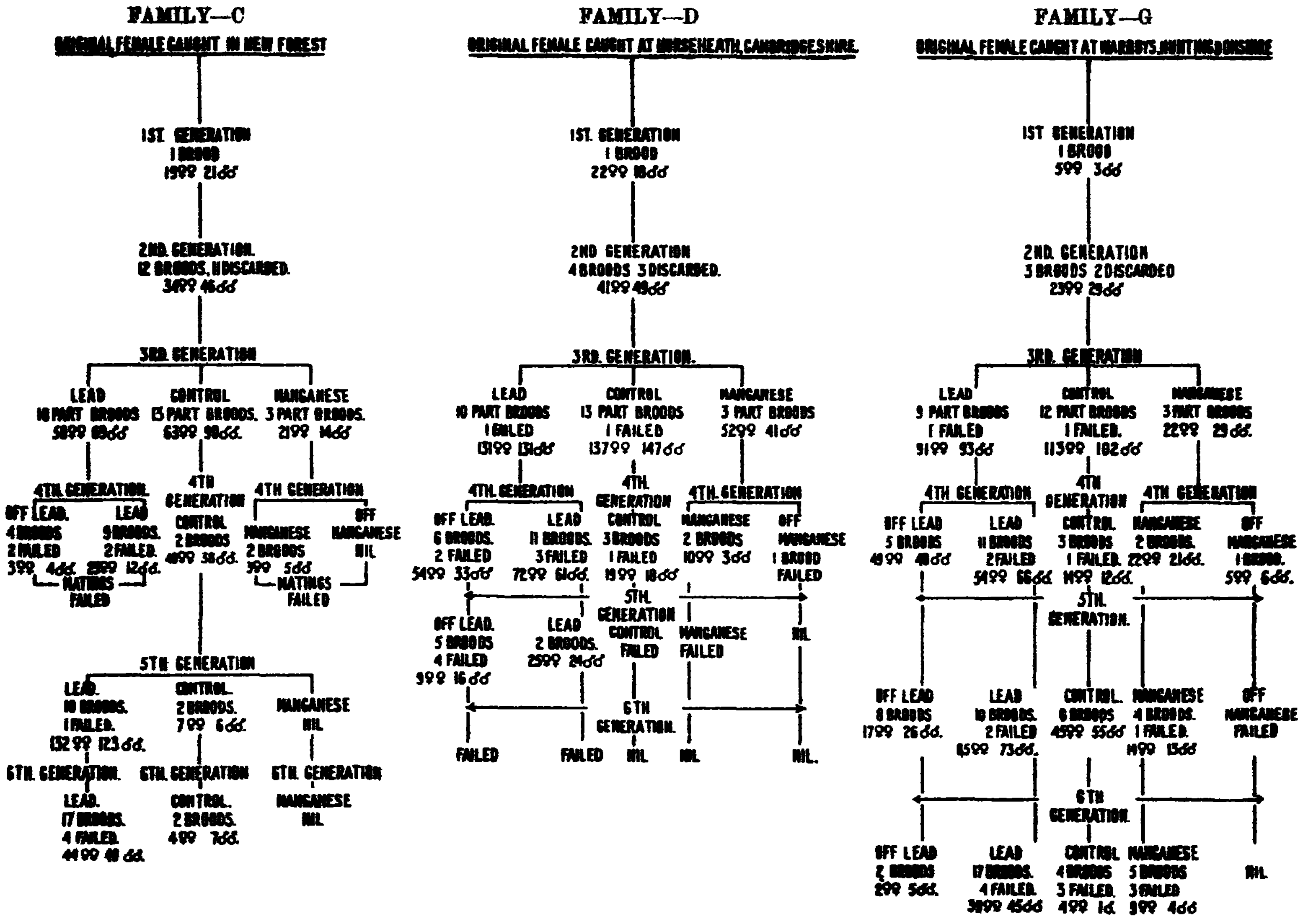
The same metallic salts as those used by Professor Harrison and Dr. Garrett were chosen for this experiment, lead nitrate and manganous sulphate. Cut twigs of hawthorn were placed with their ends in a solution of these salts in a concentration of 1 gramme per litre for at least 24 hours before use. Analyses were made from time to time to ascertain the metallic content of the foliage. In the first two treated generations a departure was made from Harrison and Garrett's method as the twigs, having been treated for not less than 24 hours, were placed in ordinary water in the breeding cylinders. In the last two treated generations the solution was also placed in the cylinders.

The Experiments

The families retained were, C from the New Forest, D from Horseheath, Cambridgeshire, and G from Warboys, Huntingdonshire. After the first two inbred generations in which only pure untreated hawthorn was used a third generation was raised and it was decided to divide each brood into treated and control portions and to limit the controls to 50 larvæ. It was further thought that 10 lead-treated and 3 manganese-treated broods per family with their controls would be sufficient to serve as a basis for a larger fourth generation. Each brood was therefore divided and the family letter preceded the treatment letter, L meaning lead, M meaning manganese, and N meaning " nothing " or control, over the index of the year and whether Spring or July broods, S or J. Thus the first lead-treated broods were called $\frac{CL1}{29S}$, $\frac{DL1}{29S}$, $\frac{GL1}{29S}$, their controls were called $\frac{CN1}{29S}$, and so on respectively. The first manganese broods were called $\frac{CM1}{29S}$, $\frac{DM1}{29S}$, $\frac{GM1}{29S}$, their controls were differentiated from the lead controls by having alpha, beta, and gamma after them, thus $\frac{CN1\alpha}{29S}$, $\frac{DN1\beta}{29S}$, $\frac{GN1\gamma}{29S}$. In succeeding generations it was always hoped to keep at least two control broods for each family, but this was not always possible.

It will be noticed that as the families were progressively inbred their viability decreased. In the first generation from wild-collected females 107 eggs yielded 88 imagines; in the sixth generation, the one family that had been preserved in unbroken descent yielded only 109 imagines from 2497 eggs (sum of the treated and control broods). There is no evidence that the treatment had given rise to any difference of viability. In the fifth generation, for which

EXPERIMENT IN MELANISM IN *SELENIA BILUNARIA*, Esper


FAMILY—C
ORIGINAL FEMALE CAUGHT IN NEW FOREST
1ST. GENERATION 1 BROOD 19♀♀ 21♂♂
2ND. GENERATION. 12 BROODS, 11 DISCARDED. 34♀♀ 46♂♂
3RD. GENERATION
LEAD 16 PART BROODS 58♀♀ 69♂♂
CONTROL 15 PART BROODS. 63♀♀ 90♂♂.
MANGANESE 3 PART BROODS. 21♀♀ 14♂♂
4TH. GENERATION.
OFF LEAD. 4 BROODS 2 FAILED 3♀♀ 4♂♂.
LEAD 9 BROODS. 2 FAILED. 25♀♀ 12♂♂.
MATINGS FAILED
4TH GENERATION CONTROL 2 BROODS 40♀♀ 38♂♂.
4TH GENERATION
MANGANESE 2 BROODS 3♀♀ 5♂♂
OFF MANGANESE NIL
MATINGS FAILED
5TH GENERATION
LEAD. 10 BROODS. 1 FAILED. 132♀♀ 123♂♂.
CONTROL. 2 BROODS. 7♀♀ 6♂♂.
MANGANESE NIL
6TH. GENERATION. LEAD. 17 BROODS. 4 FAILED. 44♀♀ 48♂♂.
6TH. GENERATION CONTROL. 2 BROODS. 4♀♀ 7♂♂.
6TH. GENERATION MANGANESE NIL
FAMILY—D
ORIGINAL FEMALE CAUGHT AT HORSEHEATH, CAMBRIDGESHIRE.
1ST. GENERATION 1 BROOD 22♀♀ 18♂♂
2ND GENERATION 4 BROODS 3 DISCARDED. 41♀♀ 48♂♂
3RD. GENERATION.
LEAD 10 PART BROODS 1 FAILED 131♀♀ 131♂♂
CONTROL 13 PART BROODS 1 FAILED 137♀♀ 147♂♂
MANGANESE 3 PART BROODS 52♀♀ 41♂♂
4TH. GENERATION
OFF LEAD. 6 BROODS. 2 FAILED 54♀♀ 33♂♂
LEAD 11 BROODS. 3 FAILED 72♀♀ 61♂♂.
4TH. GENERATION CONTROL 3 BROODS 1 FAILED 19♀♀ 18♂♂
4TH. GENERATION
MANGANESE 2 BROODS 10♀♀ 3♂♂
OFF MANGANESE 1 BROOD FAILED
OFF LEAD. 5 BROODS 4 FAILED 9♀♀ 16♂♂
LEAD 2 BROODS. 25♀♀ 24♂♂
5TH. GENERATION CONTROL FAILED
MANGANESE FAILED
NIL
6TH GENERATION.
FAILED
FAILED
NIL
NIL
NIL.
FAMILY—G
ORIGINAL FEMALE CAUGHT AT WARBOYS, HUNTINGDONSHIRE
1ST GENERATION 1 BROOD 5♀♀ 3♂♂
2ND. GENERATION 3 BROODS 2 DISCARDED 23♀♀ 29♂♂
3RD. GENERATION
LEAD 9 PART BROODS 1 FAILED 91♀♀ 93♂♂
CONTROL 12 PART BROODS 1 FAILED. 113♀♀ 102♂♂
MANGANESE 3 PART BROODS 22♀♀ 29♂♂.
4TH GENERATION
OFF LEAD 5 BROODS 49♀♀ 48♂♂
LEAD 11 BROODS 2 FAILED 54♀♀ 66♂♂
4TH GENERATION CONTROL. 3 BROODS 1 FAILED. 14♀♀ 12♂♂.
4TH GENERATION
MANGANESE 2 BROODS. 22♀♀ 21♂♂.
OFF MANGANESE 1 BROOD. 5♀♀ 6♂♂.
5TH. GENERATION.
OFF LEAD 8 BROODS 17♀♀ 26♂♂.
LEAD 10 BROODS. 2 FAILED 65♀♀ 73♂♂.
CONTROL. 6 BROODS 45♀♀ 55♂♂
MANGANESE 4 BROODS. 1 FAILED. 14♀♀ 15♂♂
OFF MANGANESE FAILED
6TH GENERATION.
OFF LEAD 2 BROODS 2♀♀ 5♂♂.
LEAD 17 BROODS. 4 FAILED. 38♀♀ 45♂♂
CONTROL 4 BROODS 3 FAILED. 4♀♀ 1♂.
MANGANESE 5 BROODS 3 FAILED 9♀♀ 4♂♂
NIL.

greater numbers are available, the untreated controls yielded 100 imagines from 781 eggs (12 8 per cent), the lead-treated broods yielded 138 imagines from 960 eggs (14 3 per cent), the manganese-treated broods yielded 27 imagines from 325 eggs (8 3 per cent) In only one instance was disease seen, and the affected brood was at once eliminated and the cages disinfected throughout

The progress of the experiment in the different families can best be followed in table form which follows

Family C

Generation.	Brood	Origin.	Treatment	No of ova.	No of larvae	No of pupae	No of females	No of males	No of matings	Matings discarded	Matings failed	Remarks
1st	C	Wild female from New Forest	0	46	45	40	19	21	12	7	4	Typical Juliana insects
2nd	C 11	C	0	200	100	85	34	46	27	11	3	Typical Spring insects
3rd	CL1 298	C 11	Lead	145	18	10	3	6	2	—	—	—
	CN1 298		Control		18	8	3	4	—	—	—	—
3rd	CL2 298	C 11	Lead	114	30	20	7	12	—			—
	CN2 298		Control		29	21	12	9	2	2	—	
3rd	CL3 298	C 11	Lead	123	12	8	5	2	2	—	-	—
	CN3 298		Control		11	7	3	2	—	-	—	-
3rd	CL4 298	C 11	Lead	94	25	19	10	8	4	—	—	—
	CN4 298		Control		22	17	6	9	2	—	—	—
3rd	CL5 298	C 11	Lead	136	6	4	—	3	—	—	—	—
	CN5 298		Control		5	2	1	1	—	—	—	—

Family C—(continued)

Generation.	Brood	Origin	Treatment.	No of ova	No of larvae	No of pupae.	No of females	No of males.	No of matings	Matings discarded	Matings failed.	Remarks
3rd	CL6 / 298	C 11	Lead	125	24	12	3	9	2	—	—	—
	CN6 / 298		Control		23	9	5	3	—	—	—	—
3rd	CL7 / 298	C 11	Lead	98	23	10	3	7	2	—		
	CN7 / 298		Control		23	19	3	14	—	—	—	
3rd	CL8 / 298	C 11	Lead	116	12	8	6	2	1	—	—	
	CN8 / 298		Control		11	9	5	4	—	—	—	—
3rd	CL9 / 298	C 11	Lead	155	29	13	5	8	2	—	—	—
	CN9 / 298		Control		28	16	5	11	—	—	—	
3rd	CL10 / 298	C 11	Lead	149	30	21	8	12	2	—		—
	CN10 / 298		Control		30	12	4	6	—	—	—	
3rd	CM1 / 298	C 11	Manganese	133	17	7	6	1	1	—	—	
	CN1a / 298		Control		17	8	3	4	—		—	
3rd	CM2 / 298	C 11	Manganese	138	21	7	5	1	1	—	—	—
	CN2a / 298		Control		20	13	6	7	—	—	—	—
3rd	CM3 / 298	C 11	Manganese	135	41	24	10	12	5	—	—	—
	CN3a / 298		Control		40	22	7	15	—	—	—	—

Family C—(continued)

Generation.	Brood	Origin	Treatment	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded	Matings failed	Remarks
4th	CL1 29J	CL3 29S	0	70	15	6	1	3	—	-	—	1 female malformed, discarded
4th	CL2 29J	CL3 29S	0	100	12	3	2	1	1	-	—	—
4th	CL3 29J	CL4 29S	Lead	99	9	2	1	1	—	—		-
4th	CL4 29J	CL8 29S	Lead	85	14	13	7	5	4	-	—	2 females malformed
4th	CL5 29J	CL4 29S	Lead	149	28	11	4	5	2	—	-	1 male malformed, discarded
4th	CL6 29J	CL6 29S	Lead	108	29	6	5	1	1	-	—	1 female malformed, discarded
4th	CL7 29J	CL1 29S	0	75	9	—	—	—	—	—	-	All died
4th	CL8 29J	CL6 29S	0	55	1	—	—	—	—	—	-	Died
4th	CL9 29J	CL2 29S	Lead	65	2	—	-	—	-	—	--	Died
4th	CL10 29J	CL4 29S	Lead	87	4	1	1	—	—	—		-
4th	CL11 29J	CL9 29S	Lead	112	24	1	1	—	-	—	—	—
4th	CL12 29J	CL10 29S	Lead	75	12	7	4	—	—	-	-	-
4th	CL13 29J	CL9 29S	Lead	124	6	—	—	—	—	—	—	All died
4th	CN1 29J	CN4 29S	Control	136	117	89	39	36	13	—	—	-

Family C—(continued)

Generation	Brood	Origin	Treatment	No of ova	No of larvæ	No of pupæ	No of females	No of males.	No of matings	Matings discarded	Matings failed	Remarks
4th	CN2 29J	CN2 29S	Control	83	5	3	1	2			--	1 female malformed, discarded
4th	CM1 29J	CM1 29S	Manganese	42	5	4	1	1		—		
4th	CM2 29J	CM2 29S	Manganese	109	21	7	2	4	2	—	2	-
5th	CL1 30S	CN1 29J	Lead	114	32	—	—	—	—	—	-	All died
5th	CL2 30S	CN1 29J	Lead	92	23	15	9	6	4	1	3	—
5th	CL3 30S	CN1 29J	Lead	162	108	89	45	38	9	—	3	—
5th	CL4 30S	CN1 29J	Lead	107	57	36	17	19	5		2	—
5th	CL5 30S	CN1 29J	Lead	109	53	44	22	22	10	—	5	—
5th	CL6 30S	CN1 29J	Lead	127	63	26	10	14	4	2	2	—
5th	CL7 30S	CN1 29J	Lead	62	25	1	1	—	—	—	--	—
5th	CL8 30S	CN1 29J	Lead	60	18	18	6	12	—	—	—	-
5th	CL9 30S	CN1 29J	Lead	87	36	11	4	6	1	—	1	
5th	CL10 30S	CN1 29J	Lead	139	103	40	18	6	3	—	3	--
5th	CN1 30S	CN1 29J	Control	105	44	11	5	6	4	2	—	—
5th	CN2 30S	CN1 29J	Control	89	20	2	2	—	—	—	—	-

Family C—(continued)

Generation	Brood	Origin	Treatment	No of ova.	No of larvæ.	No of pupæ.	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks
6th	CL1/30J	CL5/30S	Lead	137	121	11	6	5	1	—	—	Very variable temperature during larval period
6th	CL2/30J	CL5/30S	Lead	146	69	3	1	2	—	—	—	Very variable temperature during larval period
6th	CL3/30J	CL3/30S	Lead	101	61	7	4	2	1	—	—	Very variable temperature during larval period
6th	CL4/30J	CL3/30S	Lead	119	76	10	6	4	1	—	1	Very variable temperature during larval period
6th	CL5/30J	CL5/30S	Lead	156	41	16	7	7	1	—	1	Very variable temperature during larval period
6th	CL6/30J	CL5/30S	Lead	97	49	5	1	3	—	—	—	Very variable temperature during larval period
6th	CL7/30J	CL3/30S	Lead	122	57	1	—	1	—	—	—	Very variable temperature during larval period
6th	CL8/30J	CL3/30S	Lead	106	61	8	2	6	1	—	1	Very variable temperature during larval period
6th	CL9/30J	CL5/30S	Lead	71	21	2	2	—	—	—	—	Very variable temperature during larval period
6th	CL10/30J	CL4/30S	Lead	137	48	11	3	8	—	—	—	Very variable temperature during larval period
6th	CL11/30J	CL4/30S	Lead	114	53	1	—	—	—	—	—	Died, very variable temperature during larval period
6th	CL12/30J	CL3/30S	Lead	118	106	17	9	7	1	—	1	—
6th	CL13/30J	CL4/30S	Lead	142	62	4	1	3	—	—	—	—
6th	CL14/30J	CL6/30S	Lead	98	34	—	—	—	—	—	—	All died

Family C—(continued)

Generation.	Brood	Origin.	Treatment.	No of ova.	No of larvæ.	No of pupæ.	No of females.	No of males.	No. of matings.	Matings discarded.	Matings failed.	Remarks
6th	CL15/30J	CL3/30S	Lead	96	31	4	2	1	1	—	1	-
6th	CL16/30J	CL6/30S	Lead	111	81	-	—	—	—	—	—	All died
6th	CL17/30J	CL2/30S	Lead	116	51	—	—	—	—	—		All died
6th	CN1/30J	CN1/30S	Control	91	25	9	3	5	2	—	2	—
6th	CN2/30J	CN1/30S	Control	103	51	3	1	2	1	—	1	—

Family D

Generation.	Brood	Origin.	Treatment.	No of ova.	No of larvæ.	No of pupæ.	No of females.	No of males.	No. of matings.	Matings discarded.	Matings failed.	Remarks
1st	D	Wild female from Horseheath	0	51	51	44	22	18	7	6	—	Typical Juliaria insects
2nd	D4	D	0	189	120	97	41	49	26	13	—	Typical Spring insects
3rd	DL1/29S	D 4	Lead	181	96	67	26	32	5	—	1	
	DN1/29S		Control		50	39	17	21	—	—	—	-
3rd	DL2/29S	D 4	Lead	191	41	38	22	16	6	—	2	—
	DN2/29S		Control		41	28	13	15	1	—	—	-
3rd	DL3/29S	D 4	Lead	201	59	25	10	10	4	—	2	—
	DN3/29S		Control		50	24	11	12	—	—	—	

Family D—(continued)

Generation.	Brood.	Origin	Treatment	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings.	Matings discarded	Matings failed.	Remarks.
3rd	DL4/298	D 4	Lead	202	71	29	12	14	3	—	1	—
	DN4/298		Control		50	20	11	8	1	—		--
3rd	DL5/298	D 4	Lead	178	37	26	14	12	3			
	DN5/298		Control		37	28	15	13				—
3rd	DL6/298	D 4	Lead	199	93	32	16	15	1	-	—	-
	DN6/298		Control		50	22	11	11	-			--
3rd	DL7/298	D 4	Lead	146	36	—	—	—		-	—	Given to chemist to estimate lead content
	DN7/298		Control		35		—	-	-	--		
3rd	DL8/298	D 4	Lead	189	80	42	19	22	1		1	--
	DN8/298		Control		50	31	15	14	—	—		—
3rd	DL9/298	D 4	Lead	178	37	21	11	9	1	—	1	—
	DN9/298		Control		37	27	14	12	—	—	-	—
3rd	DL10/298	D 4	Lead	77	3	3	1	1	—		—	—
	DN10/298		Control		2	2	—	2	—	—	--	—
3rd	DM1/298	D 4	Manganese	195	21	14	8	4	2	—	1	—
	DN1β/298		Control		27	8	3	5	—	—	—	—

Family D—(continued).

Generation.	Brood	Origin	Treatment	No of ova	No of larvæ	No of pupæ	No of females	No of males.	No of matings	Matings discarded	Matings failed	Remarks
3rd	DM2/29S	D 4	Manganese	127	43	29	19	10	6	4	–	--
	DN2β/29S		Control		43	25	16	10	1	—	—	—
3rd	DM3/29S	D 4	Manganese	235	98	59	25	27	1	—	1	—
	DM3β/29S		Control		50	40	11	24		—	—	—
4th	DL1/29J	DL4/29S	0	77	12	5	3	1	—		—	—
4th	DL2/29J	DL2/29S	0	113	—	—	--	-	-	—	—	Failed
4th	DL3/29J	DL3/29S	Lead	94	4	1	1	-	—	--	—	-
4th	DL4/29J	DL3/29S	Lead	153	32	10	5	5	1	-	1	-
4th	DL5/29J	DL5/29S	Lead	78	-	—	—	—	-	—	--	Failed
4th	DL6/29J	DL2/29S	Lead	150	29	13	4	11	2	—	2	-
4th	DL7/29J	DL1/29S	0	175	106	50	28	14	13	—	11	--
4th	DL8/29J	DL1/29S	0	172	106	—	—	—	—	—	—	Mixed with DL9/29J, discarded
4th	DL9/29J	DL2/29S	Lead	130	60	—	—	—	—	—	—	Mixed with DL8/29J, discarded
4th	DL10/29J	DL5/29S	Lead	121	54	26	12	14	9	—	9	—

Family D—(continued)

Generation	Brood.	Origin.	Treatment.	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded	Matings failed.	Remarks
4th	DL11/29J	DL1/29S	Lead	149	78	46	23	17	6	—	5	—
4th	DL12/29J	DL5/29S	Lead	121	43	8	5	2	2	—	2	—
4th	DL13/29J	DL1/29S	0	152	63	31	16	15	12	—	9	—
4th	DL14/29J	DL4/29S	Lead	79	14	-	—	—	—	—	—	All died
4th	DL15/29J	DL2/29S	Lead	125	37	21	8	8	5	—	5	—
4th	DL16/29J	DL9/29S	0	156	48	11	7	3	2	—	2	—
4th	DL17/29J	DL6/29S	Lead	115	79	22	14	4	4	—	3	—
4th	DN1/29J	DN2/29S	Control	134	65	43	14	17	5	—	5	—
4th	DN2/29J	DN4/29S	Control	94	4	—	—	—	—	—	—	Discarded, diseased.
4th	DN3/29J	DN2/29S	Control	105	40	10	5	1	—	—	—	—
4th	DM1/29J	DM2/29S	Manganese	157	22	8	5	2	1	—	1	—
4th	DM2/29J	DM2/29S	Manganese	145	10	7	5	1	1	1	—	—
4th	DM3/29J	DM1/29S	0	80	16	—	—	—	—	—	—	All died
5th	DL1/30S	DL7/29J	0	95	46	29	9	16	4	—	4	—

Family D—(continued)

Generation	Brood	Origin	Treatment	No of ova	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks.
5th	DL2/30S	DL13/29J	0	92	—	—	—	—	—	—	—	Failed
5th	DL3/30S	DL13/29J	0	127	33	—	—	—	-	--	-	All died
5th	DL4/30S	DL7/29J	0	34	—	—	—	—	—	—	—	Failed
5th	DL5/30S	DL17/29J	Lead	90	39	35	17	18	9	—	7	—
5th	DL6/30S	DL13/29J	0	128	15	—		-	-	-	-	All died
5th	DL7/30S	DL11/29J	Lead	46	27	15	8	6	3	—	3	—
6th	DL1/30J	DL5/30S	Lead	39	2	—	—	-	—	—	—	Died.
6th	DL2/30J	DL5/30S	Lead	53	3	—	—		- -	—	—	Died

Family G

Generation	Brood	Origin	Treatment	No of ova	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks.
1st	G	Wild female from Warboys	0	10	10	8	5	3	3	2	—	Typical Juliaria insects
2nd	G 3	G	0	199	113	71	23	29	17	2	3	Typical Spring insects.
3rd	GL1/29S	G 3	Lead	128	27	13	7	5	—	—	—	—
	GN1/29S		Control		26	15	8	7	—	—	—	—

Family G—(continued)

Generation	Brood	Origin.	Treatment	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks
3rd	GL2 / 298	G 3	Lead	153	47	21	11	8	4	—	1	—
	GN2 / 298		Control		46	27	11	15	2	1	—	—
3rd	GL3 / 298	G 3	Lead	143	43	31	16	14	4	—	-	—
	GN3 / 298		Control		43	31	15	14	1			-
3rd	GL4 / 298	G 3	Lead	174	92	46	19	21	4	—	—	—
	GN4 / 298		Control		50	25	11	13	-			-
3rd	GL5 / 298	G 3	Lead	156	28	21	10	11	3		1	—
	GN5 / 298		Control		27	21	7	12			-	—
3rd	GL6 / 298	G 3	Lead	157	18	—	—	—	—	—	—	Given to chemist to estimate lead content
	GN6 / 298		Control		18	—	-	—	—	-		
3rd	GL7 / 298	G 3	Lead	184	31	19	8	11	1	—	—	—
	GN7 / 298		Control		30	18	12	5	—	—	—	—
3rd	GL8 / 298	G 3	Lead	104	13	11	4	6	1	—	—	—
	GN8 / 298		Control		13	8	5	3	—	—		—
3rd	GL9 / 298	G 3	Lead	171	40	34	16	17	1	—	—	—
	GN9 / 298		Control		40	26	14	11	1	—	-	—

Family G—(continued)

Generation.	Brood	Origin	Treatment	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded	Matings failed.	Remarks
3rd	GM1/298	G 3	Manganese	113	32	17	6	11	2	–	—	—
	GN1γ/298		Control		32	19	11	7	–	—	—	—
3rd	GM2/298	G 3	Manganese	156	63	25	10	13	1	—		—
	GN2γ/298		Control		50	23	13	9	—	—		—
3rd	GM3/298	G 3	Manganese	195	17	11	6	5	—	—	–	–
	GN3γ/298		Control		17	13	6	6	–	—	—	—
4th	GL1/29J	GL2/298	0	139	39	8	5	3	3	—	3	—
4th	GL2/29J	GL3/298	Lead	119	43	1	–		—		– –	This family died very rapidly, and the one pupa failed to emerge
4th	GL3/29J	GL2/298	Lead	138	68	8	5	3	3	—	2	Died rapidly in larval stage
4th	GL4/29J	GL3/298	0	146	36	9	6	2	2	--	-	-
4th	GL5/29J	GL3/298	Lead	136	42	—	–		—	—	—	Failed, all died.
4th	GL6/29J	GL4/298	Lead	158	72	9	3	5	2	-	—	—
4th	GL7/29J	GL4/298	0	173	92	32	16	15	9	—	5	—
4th	GL8/29J	GL3/298	Lead	146	37	10	2	7	1	--	1	—
4th	GL9/29J	GL2/298	Lead	118	30	4	4	—	—	—	—	—

Family G—(continued)

Generation.	Brood	Origin	Treatment	No of ova.	No of larvæ.	No of pupæ	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks
4th	GL10 29J	GL4 29S	0	181	129	44	22	18	9	—	7	—
4th	GL11 29J	GL4 29S	Lead	138	55	28	12	14	7		4	-
4th	GL12 29J	GL5 29S	Lead	117	23	14	4	10	3	-	3	--
4th	GL13 29J	GL5 29S	0	145	42	2	–	2	--	—	-	—
4th	GL14 29J	GL7 29S	Lead	181	72	24	10	11	6	—	5	–
4th	GL15 29J	GL9 29S	Lead	140	78	19	8	9	4	–	3	—
4th	GL16 29J	GL8 29S	Lead	144	74	15	6	7	3	-	1	—
4th	GM1 29J	GM1 29S	Manganese	164	117	42	19	19	6	—	2	–
4th	GM2 29J	GM1 29S	Manganese	121	53	7	3	2	1	—	1	—
4th	GM3 29J	GM2 29S	0	139	91	16	5	6	4	—	4	—
4th	GN1 29J	GN2 29S	Control	172	--	—	—	—	—		-	Infertile failed
4th	GN2 29J	GN3 29S	Control	125	95	2	1	—	—	—	—	Very hot weather July 22–30, many died in larval stage.
4th	GN3 29J	GN9 29S	Control	150	88	30	13	12	10	—	4	—
5th	GL1 30S	GL6 29J	Lead	77	24	—	—	—	—	—	—	All died

Family G—(continued)

Generation.	Brood.	Origin.	Treatment	No of ova.	No of larvæ	No of pupæ	No of females	No of males	No of matings	Matings discarded.	Matings failed	Remarks
5th	GL2 30S	GL3 29J	Lead	96	35	22	8	14	7	—	3	-
5th	GL3 30S	GL4 29J	0	52	3	2	—	1	-	—	—	—
5th	GL4 30S	GL4 29J	0	128	26	6	3	3	2	—	—	—
5th	GL5 30S	GL6 29J	Lead	97	32	2	—	1	—	—	--	—
5th	GL6 30S	GL11 29J	Lead	111	62	23	7	11	5	—	2	—
5th	GL7 30S	GL11 29J	Lead	55	44	32	15	14	10	—	8	—
5th	GL8 30S	GL7 29J	0	122	30	1	1	—	—	—	—	—
5th	GL9 30S	GL7 29J	0	115	50	3	—	2	—	—	—	—
5th	GL10 30S	GL7 29J	0	50	15	6	4	2	1	-	1	—
5th	GL11 30S	GL15 29J	Lead	149	49	31	16	14	8	—	6	—
5th	GL12 30S	GL11 29J	Lead	151	39	21	11	8	7	—	5	—
5th	GL13 30S	GL10 29J	0	85	34	7	1	5	—	—	—	—
5th	GL14 30S	GL10 29J	0	165	46	15	6	6	4	—	4	—
5th	GL15 30S	GL7 29J	0	180	59	10	2	7	—	—	—	—

Family G—(continued).

Generation.	Brood.	Origin.	Treatment.	No of ova.	No of larvæ.	No of pupæ.	No of females.	No of males.	No of matings.	Matings discarded.	Matings failed.	Remarks
5th	GL16 30S	GL14 29J	Lead	107	26	6	—	4	—	—	—	—
5th	GL17 30S	GL16 29J	Lead	97	35	10	8	7	6	—	3	—
5th	GL18 30S	GL16 29J	Lead	20	6	-	—		—	—	-	All died
5th	GN1 30S	GN3 29J	Control	133	28	16	6	9	5	—	5	—
5th	GN2 30S	GN3 29J	Control	122	23	15	5	9	1	—	1	—
5th	GN3 30S	GN3 29J	Control	149	30	20	10	10	2	—	-	—
5th	GN4 30S	GN3 29J	Control	130	31	23	8	15	7	—	6	—
5th	GN5 30S	GN3 29J	Control	91	19	7	4	3	1	—	1	—
5th	GN6 30S	GN3 29J	Control	156	26	21	12	9	4	—	3	—
5th	GM1 30S	GM1 29J	Manganese	63	38	—	—	—	—	—	—	All died
5th	GM2 30S	GM1 29J	Manganese	111	32	12	7	4	2	—	1	—
5th	GM3 30S	GM1 29J	Manganese	53	18	11	4	7	3	—	—	—
5th	GM4 30S	GM1 29J	Manganese	98	16	5	3	2	1	—	—	—
6th	GL1 30J	GL2 30S	Lead	136	32	1	1	—	—	—	—	—

Family G—(continued).

Generation.	Brood.	Origin.	Treatment.	No of ova.	No of larvæ.	No of pupæ.	No of females.	No of males.	No of matings.	Matings discarded.	Matings failed.	Remarks.
6th	GL2/30J	GL7/30S	Lead	80	33	—	—	—	—	—	—	All died
6th	GL3/30J	GL4/30S	0	143	95	2	1	1	—	-	—	—
6th	GL4/30J	GL2/30S	Lead	74	15	1	—	1	—	-	-	—
6th	GL5/30J	GL2/30S	Lead	87	51	3	1	—	--	—	—	—
6th	GL6/30J	GL6/30S	Lead	65	14	—	—	—	—	—	—	All died
6th	GL7/30J	GL2/30S	Lead	22	—	—	—	—	—	-		Failed.
6th	GL8/30J	GL7/30S	Lead	85	33	13	4	6	1	—	1	—
6th	GL9/30J	GL6/30S	Lead	88	61	9	5	2	1	—	1	.
6th	GL10/30J	GL4/30S	0	169	102	6	1	4	1	-	1	—
6th	GL11/30J	GL6/30S	Lead	99	37	14	8	4	1	—	1	—
6th	GL12/30J	GL11/30S	Lead	94	47	5	1	4	—	—	—	—
6th	GL13/30J	GL12/30S	Lead	99	78	3	2	1	1	—	—	—
6th	GL14/30J	GL17/30S	Lead	113	46	7	3	4	2	—	2	—
6th	GL15/30J	GL5/30S	Lead	126	116	34	13	20	4	—	2	—

Family G—(continued).

Generation.	Brood	Origin	Treatment	No of ova	No of larvæ	No of pupæ.	No. of females	No. of males	No of matings.	Matings discarded.	Matings failed.	Remarks
6th	$\frac{\text{GL16}}{\text{30J}}$	$\frac{\text{GL11}}{\text{30S}}$	Lead	29	20	2	—	2	—	—	—	—
6th	$\frac{\text{GL17}}{\text{30J}}$	$\frac{\text{GL17}}{\text{30S}}$	Lead	67	41	1	—	1	—	—	—	—
6th	$\frac{\text{GL18}}{\text{30J}}$	$\frac{\text{GL17}}{\text{30S}}$	Lead	114	40	4	1	—	—	—	—	—
6th	$\frac{\text{GL19}}{\text{30J}}$	$\frac{\text{GL12}}{\text{30S}}$	Lead	39	20	—	—	—	—	—	—	All died
6th	$\frac{\text{GN1}}{\text{30J}}$	$\frac{\text{GN3}}{\text{30S}}$	Control	98	38	6	4	1	1	—	1	—
6th	$\frac{\text{GN2}}{\text{30J}}$	$\frac{\text{GN4}}{\text{30S}}$	Control	109	44	3	-	—	—	—	—	Died
6th	$\frac{\text{GN3}}{\text{30J}}$	$\frac{\text{GN6}}{\text{30S}}$	Control	44	33	—	—	—	—	—	—	All died
6th	$\frac{\text{GN4}}{\text{30J}}$	$\frac{\text{GN3}}{\text{30S}}$	Control	41	29	—	—	—	—	—	—	All died
6th	$\frac{\text{GM1}}{\text{30J}}$	$\frac{\text{GM3}}{\text{30S}}$	Manganese	98	38	—		—	—	—	—	All died
6th	$\frac{\text{GM2}}{\text{30J}}$	$\frac{\text{GM2}}{\text{30S}}$	Manganese	89	70	—	—	—	—	—	—	All died.
6th	$\frac{\text{GM3}}{\text{30J}}$	$\frac{\text{GM3}}{\text{30S}}$	Manganese	124	66	13	7	4	2	—	2	—
6th	$\frac{\text{GM4}}{\text{30J}}$	$\frac{\text{GM3}}{\text{30S}}$	Manganese	111	74	2	2	—	—	—	—	—
6th	$\frac{\text{GM5}}{\text{30J}}$	$\frac{\text{GM4}}{\text{30S}}$	Manganese	54	18	—	—	—	—	—	—	All died.

Analyses

In the first year (1929) of feeding on treated foliage, analyses were made in order to ascertain that the lead and manganese did reach the leaves and the larvæ In the untreated foliage the amount of lead was never measurable, but manganese was always present in amounts varying from 0 0003 per cent early in the season to 0 002 per cent in August. In the treated foliage the lead was always present and varied between 0·002 and 0 006 per cent The manganese content was also increased, on occasion, to 0·004 and 0 005 per cent. These percentages are calculated on the green material

Because of the difficulty of accurate estimation of these very minute quantities of lead and manganese, at the instance of the Ministry of Agriculture, Sir Robert Robertson was good enough to undertake some further analyses by the refined methods employed in the Government Laboratory Leaves had been preserved at three dates, May, July, and August, 1930, the untreated material yielded respectively 0 009, 0 008 and 0 011 mgm of lead per gram of dry material The corresponding treated material contained 0 24, 0 20, and 0·092 mgm of lead per gram Of the manganese the untreated material yielded 0·05, 0 05, and a trace, the treated material gave 1 1, 0 5, and 4 4 mgm per gram of dry leaf The imagines reared in the third and fourth generations and also subsequently those of the sixth generation were analysed, but the amounts of lead and manganese found were too minute to be able to say conclusively that there was more lead or manganese in the treated moths than in those which were untreated

Conclusion

Whereas Heslop Harrison obtained melanic moths in the second treated generation, no melanism has appeared in the present experiment even after four treated generations The final imagines obtained are the sixth inbred generation since the start of the experiment in 1928 In this experiment therefore the attempt to induce melanism in *Selenia bilunaria*, Esper, by treatment with lead nitrate and manganous sulphate has failed

Summary

(1) An attempt has been made to induce melanism in *Selenia bilunaria*, Esper, by feeding the larvæ on hawthorn treated with lead and manganese salts

(2) Six generations totalling 3265 moths have been reared

(3) No melanic individuals have appeared either in the treated or the control broods

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Professor J B S Haldane has kindly contributed the following discussion

The system of matings adopted was designed to reveal any recessive factors for melanism latent in either control or experimental stocks Had melanism appeared in the latter and not the controls, the latter were sufficiently numerous to make it almost certain that it was due to mutation If we call the recessive factor for melanism *t*, the corresponding dominant T (Harrison's terminology), the mating of T*t* × T*t* gives about a quarter melanics Thus if a family of n is bred, the chance of obtaining at least one melanic is $1 - (\frac{3}{4})^n$. For example, if $n = 10$ this chance is 0 9437, *i e*, only 5 6 per cent of such families should fail to reveal the recessive factor

The 82 broods, some of whose ancestors had been treated with lead, contained 910 individuals, none of which were melanic. Harrison and Garrett record five similar broods (1923 AL, BL, LL and TL, and 1924 LL) which contained 136 types and 6 melanics We can arrange these figures with those of Hughes in a fourfold table, and find a measure of divergence $\chi = 6 \cdot 17$. The probability of so great a divergence being due to chance is less than 10^{-9}. The use of the χ^2 formula is not, of course, quite legitimate when one of the four numbers is zero, but the order of magnitude of the probability is unaffected. Had these two experiments given parallel results, 38·1 melanics would have been expected among the 910 moths reared

The comparison can be made even more strongly by comparing only broods whose parents and themselves were reared on lead-treated food. Here 652 types and no melanics were obtained, while Harrison and Garrett obtained 71 types and 6 melanics. The expectation on this basis in the Merton experiments was 50·8 melanics, and the probability that their non-appearance was due to chance was very much less than in the former case

We can compare the results in still another way In the 82 broods from lead-treated ancestry the average number per brood was 11 09, and 35 contained 10 or more individuals We may take it then that somewhat over 40 parents of these broods were not both heterozygous for the factor *t* If now the appearance of melanism was due to the heterozygosity of the parents (an hypothesis which Harrison rejects), in his case three pairs out of five were both heterozygous Here $\chi = 5\ 07$, and the probability that the divergence is due to chance is about $3\ 8 \times 10^{-7}$

The manganese results may be treated in the same way, though, being fewer, they are less conclusive Here 12 broods whose ancestors had been reared on manganese gave 115 types and no melanics In similar families Harrison and Garrett obtained 12 types and 8 melanics in one family (1923, DM) while Harrison obtained 310 types, 29 melanics, and 4 mosaics in five families (T1, T2, T3, T4, T5) Here $\chi = 3\ 12$, and the probability that the divergence is due to chance is 0 0018

It is thus perfectly clear that had Hughes' insects behaved in the same way as Harrison's, some melanics would have appeared The chances of their appearing in this case were almost a billion to one

Considering Harrison and Garrett's results, if his original female or her mate were T*t*, the other parent being TT, half the offspring would be T*t* In such a case the chances are even that the pair chosen to breed from were also TT and T*t*, giving the all-type brood of summer 1922 Again, there is a chance of one in four that the two pairs of this family chosen for breeding were TT and T*t* Controls were bred for the lead experiment, and presumably in this case two TT parents were chosen, and their offspring bred together for two or more generations. The chance of this event occurring in a half heterozygous family would be one in four Thus the odds against a mendelian explanation of Harrison and Garrett's results are, so far, fifteen to one However, as Harrison and Garrett point out, the small proportion of melanics segregating in families AL, BL and LL of 1923 is very difficult to account for on such a basis It would readily be explicable, however, if *t* were linked with a lethal gene Such a possibility cannot be denied, and would account for the small numbers found. It would also perhaps account for the absence of melanics in control families which might otherwise have revealed heterozygosis. Harrison's later results with manganese were more adequately controlled, and the odds against a mendelian explanation are now 100 to 1, unless we assume linkage with a lethal In this case the recessives should, of course, as they did, give normal mendelian results on crossing with typical insects

It would seem, then, that although Harrison's explanation of his results is very plausible, it is not conclusively demonstrated For this reason very full controls were bred by Mr Hughes, so that, had a melanic appeared, the hypothesis of its origin from a recessive factor latent in the wild moths would have been most improbable

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Investigations on Mediterranean Kala Azar VI —Canine Visceral Leishmaniasis

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(Kala Azar Commission of the Royal Society)

(Communicated by Sir Henry Dale, Sec R S —Received February 3, 1932)

[Plates 19 and 20]

Following the discovery of canine visceral Leishmaniasis in Tunis by Nicolle and Comte (1908), an extensive literature has developed dealing particularly with the possible relationship between human and canine Leishmaniasis Most authors, following Nicolle, consider *Leishmania infantum* to be the causative organism of both human and canine Kala Azar

In the case of cutaneous Leishmaniasis, *L tropica* has been conclusively proved by direct experiment to be the causative organism in both man and dog (1930), but in this case there is no evidence that the dog serves as a reservoir of the human disease. It is more likely that where human and canine cutaneous Leishmaniasis co-exist, human beings serve as a reservoir from which dogs are infected (1929). It is, however, difficult to carry out similar direct experiments with visceral Leishmaniasis, for human adults are naturally immune to Mediterranean Kala Azar.

Canine Kala Azar is present in all considerable Mediterranean foci of infantile Kala Azar, but the epidemiological evidence relating the human to the canine disease is still incomplete, since Catania, the largest focus of infantile Kala Azar in the Mediterranean has according to Pulvirenti (1911) and Panto (1912), the lowest recorded incidence of the canine disease (1 6 per cent) Canine Kala Azar is definitely a seasonal disease, *e g* , Cardamatis (1911) in Athens found an incidence of 40 per cent in June and 7 per cent in January Sergent, Ed and Et (1910) in Algeria found an incidence of 7 2 per cent in summer, while Lemaire, Sergent and Lhéritier (1913) found an incidence of 0·97 per cent in autumn It is therefore impossible to stress the low recorded incidence in Catania until a survey on an adequate number of animals has been made for every month of the year

Up to the present the main evidence for the identity of the human and canine disease rests on animal experiments. Wenyon (1926), after reviewing the findings of many authors, states, " The disease produced in dogs by inoculation with the parasite from human cases is identical with the natural canine disease, while the organism from the canine disease is inoculable into animals with results similar to those which result from inoculation of the human virus "

The main symptoms recorded from naturally infected dogs are emaciation, anæmia, keratitis, scaling of the skin and depilation, but in some cases it is impossible to make a clinical diagnosis for, as Donatien and Lestocquard (1929) have pointed out, heavily infected animals occasionally show no signs of the disease, which may be discovered only at autopsy Changes in the skin in naturally infected dogs have been noted in all foci In Malta Critien (1911) noted that some of the infected dogs were mangy looking and had cutaneous ulcers Yakimoff and Kohl-Yakimoff (1911) in Tunis and Yakimoff (1915) in Turkestan recorded depilation in naturally infected dogs Donatien and Lestocquard (1929) noted that the areas of depilation are covered by dry scales and described the condition as one of " eczema furfurace " The same authors described small ulcers on the eczematous areas in which L D. bodies were found. Chodukin and Schevtschenko (1928) recorded cutaneous ulcers, the presence of L D bodies in the sebaceous glands or hair follicles of apparently healthy dogs with visceral Leishmaniasis in Tashkent, and compared the condition of the skin to that found in experimental Chinese hamsters. Chodukin (1928) found an infection rate of 2·8 per cent in sandflies fed on naturally infected dogs Sofieff and Schevtschenko (1929) working in Turkestan found that sandflies, *P papatasii*, became infected after feeding on dogs with mixed infections of cutaneous and visceral Leishmaniasis, but the infection rate in the insects diminished continuously from 96 per cent after one day to 4 per cent. after seven days The diminution of the infection rate in this experiment may be due to the fact that after infecting themselves with *Leishmania* the insects were allowed to feed on sugar and water If the sugar and water are not sterile this procedure inevitably produces a rapid diminution, and eventually complete destruction of the flagellates in the sandfly, owing to the multiplication of bacteria in the alimentary tract of the insects Donatien, Lestocquard and Parrot (1929) recorded a cutaneous ulcer with L D bodies in a dog with Kala Azar from Algeria, and Parrot, Donatien and Lestocquard (1930) found that 4 out of 53 sandflies, *P perniciosus*, which fed on a dog with cutaneous ulcers and Kala Azar, became infected The infection in the insects was confined to the stomach

In this paper particular stress is laid on the previous descriptions of the skin in naturally infected dogs because, as will be shown later, the changes in the skin are of fundamental importance in the pathology of canine Kala Azar and the problem of transmission by biting insects and cutaneous ulceration is not an essential or even important part of the skin condition

Findings in Malta

In Malta, Critien (1911) found 10 out of 83 dogs infected Wenyon (1914) found 6 out of 46 infected Critien found no marked variation in the incidence of the disease between April and October

Between June and October, 1931, 100 stray dogs were examined and 11 were found to be infected It was soon evident that liver puncture is not a satisfactory method of diagnosis In all cases the diagnosis was established by examining the spleen The animals were anaesthetised with a mixture of one part alcohol and three parts ether A small incision was made in the abdomen a little below the left costal margin, the upper pole of the spleen was drawn up through the incision, a small piece of spleen was removed for examination and the peritoneum, muscles and skin were stitched separately. With a little practice the whole operation should not take more than 20 minutes The operative mortality was nil and the animals showed no ill effects as a result of the operation

Nine of the 11 animals showed changes in the skin, *i e*, seborrhea and depilation Four of the animals were emaciated. The spleen was enlarged in

4, normal in 5, atrophic and fibrous in 2 cases In all the 11 animals the number of L D bodies found in spleen smears were less than that found in an average human case Plasma cells which are rare or absent in spleen smears of normal dogs were present in considerable numbers even in cases where L D bodies were scanty In the atrophic fibrous spleens L D bodies were found only after prolonged search but plasma cells were common

Histological Changes —The morbid anatomy of the viscera in canine Kala Azar has been frequently described It is only necessary to draw attention to the fibrotic changes in the spleen Fibrosis was present in four spleens which were sectioned and it is interesting to note that fibrosis was most marked in the spleens which contained least parasites Fibrosis was not seen in spleens of infected hamsters, *Cricetulus griseus* and *Cricetus auratus*, field mice, *Microtus guentheri*, and ground squirrels, *Citillus citillus* Unlike dogs, these animals never show a tendency to spontaneous cure and it is therefore suggested that the fibrosis of the spleen found in dogs is a part of the process of spontaneous cure In cases of Oriental sore in human beings spontaneous cure is always accompanied by fibrosis It is possible that infected reticulum cells become converted into connective tissue cells which are unsuitable host cells for L D bodies.

Skin —The skin changes were studied in four naturally infected animals and are described in order of the intensity of the visceral infection. Portions of the skin were examined from all parts of the body

Dog No 3 —The animal was well nourished, showed a slight amount of depilation and seborrhea L D bodies in the spleen were scanty Under a low power the skin was normal except for infiltration round some of the hair follicles, fig 1, Plate 19 The infiltration consisted almost entirely of large and small macrophages of which a few were infected with L D bodies Every section showed a few infected macrophages in the dermis quite apart from the areas of infiltration round the hair follicles There was no apparent tissue reaction round these individual infected macrophages, figs 3–4, Plate 20 The tissue changes characteristic of infections with *L tropica* in human beings and dogs, *i e*, massive infiltration with plasma cells and small round cells, were entirely absent The epidermis was normal

Dog. No 4 —The seborrhea and depilation were well marked Under the low power many of the hair follicles were found to be surrounded by areas of infiltration of the same type as in dog No 3 In some places there was infiltration of macrophages in the dermis apart from the hair follicles and the dermis was rather more cellular than that of a normal animal Although the histological changes were more pronounced, infected macrophages were fewer than in dog No 3.

Dog. No. 2.—Seborrhea and depilation were well marked and the animal was emaciated.

Many of the hair follicles were surrounded by areas of infiltration of the same type as in dog No. 3, fig 2, Plate 19. Infected macrophages were more numerous than in the previous animals both in the areas of infiltration round the hair follicles and in the normal dermis between the hair follicles Patches of dermis were rather more cellular than normal, but in the main the dermis apart from the affected hair follicles was normal A few of the hair follicles showed proliferation of epithelium from their external border The histological changes were less marked than in dog No 4, but infected macrophages were far more numerous

Dog No 1—Seborrhea and depilation were well marked The visceral infection was heavier than in all the other positive animals and spleen smears showed an average of two L D bodies per field The animal was slightly emaciated but gained weight in the laboratory The histological changes in the skin were similar to those found in dog No 2, but were rather more marked and infected macrophages were much more numerous A few plasma cells were present in the area of infiltration and were confined to the neighbourhood of the small vessels supplying the affected hair follicles. Sections of the skin showed more parasites than those of spleen or liver Infected macrophages were also found in the subcutaneous fat. The dermis in parts was more cellular than normal and there were a few areas of macrophage infiltration apart from the infected hair follicles, but many parts of the dermis were quite normal. The epidermis was normal except for a few small patches where it was thickened. Dog No 1 was observed from September 7, 1931, till November 5, 1931, when it died of broncho-pneumonia. During the last 2 weeks of the animal's life the skin condition was accompanied by irritation and several ulcers were produced as a result of scratching

Infected macrophages in the dermis of dogs are very amœboid and may send long thin pseudopodia between the connective tissue and elastic fibres among which they lie They are also phagocytic and occasionally ingest melanin granules.

The histological changes in the skin in canine Kala Azar can be briefly summarised as a selective infiltration of macrophages round hair follicles including the sebaceous glands, figs. 1–2, Plate 20, and the presence of infected macrophages in normal dermis The latter phenomenon in the complete absence of secondary infiltration of round cells and plasma cells is the most striking characteristic of canine Kala Azar and differentiates it from *L. tropica* In the more advanced stages the dermis is more cellular than that of normal dogs and may even contain a few small dense areas of infiltration with macrophages and some round cells and polymorphs, fig 3, Plate 19 The external changes, *i e*, seborrhea and depilation are roughly proportional to the number of affected hair follicles It is quite impossible to confuse the skin changes with those of *L tropica* where the infiltration of macrophages is dense and not confined to the hair follicles and the presence of infected macrophages in the dermis, particularly in the later stages, is always accompanied by considerable infiltration of plasma cells and small round cells

The histological differentiation between the skin changes in canine visceral Leishmaniasis and those in canine cutaneous Leishmaniasis are of no practical importance either in the Mediterranean basin where natural infections with *L tropica* in dogs are unknown or in Mesopotamia where natural infections with *L tropica* are common and canine Kala Azar does not occur It is, however, of the greatest importance in Turkestan, where *P sergenti*, *P papatasii* (carriers of Oriental sore) and sandflies of the *Major* group are common This, in our opinion, accounts for the fact that both cutaneous and visceral Leishmaniasis are common in man and dog in Turkestan From the description of Russian writers it is evident that mixed infections are not uncommon in dogs This fact has to be taken into account in the interpretation of transmission experiments with sandflies Chodukin, Sofieff, Schevtschenko and Radsivilovskij (1931) carried out an interesting experiment From May 10, 1930, till October 15, 1930, they kept four healthy dogs proved to be free from *Leishmania* in the same room as an infected animal in such a way that direct contact between the healthy and infected dogs was impossible During this period a total of 600 wild sandflies (*P papatasii* and *P sergenti* ♀ ♀) were introduced into the room (Both of these species have been shown experimentally to be carriers of cutaneous Leishmaniasis) On October 15, 1930, one of the healthy dogs was found to have an ulcer on the nose There were no clinical signs of visceral Leishmaniasis and no L D bodies were found in the bone marrow Since ulceration of the skin is a late manifestation of canine Kala Azar, it is very probable that the experimental animal had an infection of *L tropica.* From May 10, 1930, to about June 10, 1930, the room contained a dog naturally infected with visceral and cutaneous Leishmaniasis Subsequently, two other dogs with mixed infections of visceral and cutaneous Leishmaniasis were introduced into the room Since neither *P papatasii* nor *P sergenti* are carriers of visceral Leishmaniasis, we assume that the experimental animal was infected by a sandfly, *P. papatasii* or more probably *P sergenti*, which was either naturally infected before being introduced into the room or had infected itself during the course of the experiment by feeding on one of the dogs with a natural skin lesion. The above experiment is therefore not relevant to the problem of transmission of canine Kala Azar, but confirms the previous evidence that sandflies transmit cutaneous Leishmaniasis

Infection of Sandflies

Dog No. 1—Between September 14, 1931, and November 2, 1931 Four hundred and twenty-four females of *P perniciosus* were fed on dog No 1, and 265, *i.e.*, 62·5 per cent., became infected with *Leishmania* In the later experiments the infection rate rose occasionally up to 90 per cent

Dog No. 2.—Between September 28, 1931, and September 30, 1931 Twenty-six females of *P perniciosus* fed on dog No 2, of which 17, *i e*, 65·4 per cent, became infected

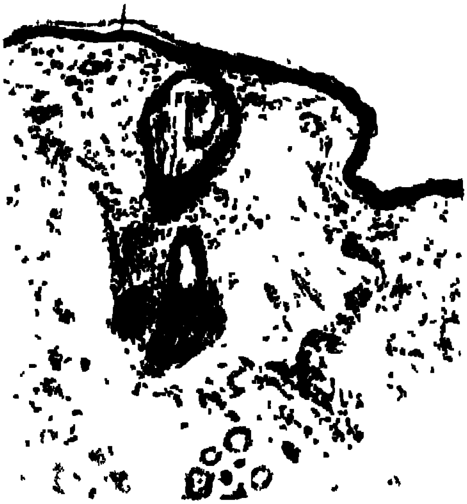
Dog No 3—Between October 10, 1931, and October 20, 1931. Thirty-four females of *P. perniciosus* fed on dog No. 3. Of these 11, *i e*, 32 per cent., became infected.

Six sandflies, *P papatasii*, fed on dog No 1 were negative for *Leishmania*

The infections persisted throughout the whole life of the sandflies. The above infection rates are much higher than that found by Parrot, Donatien and Lestocquard in June, 1930 (8 per cent.)

The behaviour of the flagellates in the infected sandflies will be discussed in another paper

Thirteen fleas, *Ctenocephalus canis*, from dog No. 2, 15 from dog No. 3, and 12 from dog No. 1, taken at a time when the infection rates in *P. perniciosus* fed on these animals were 65·4 per cent., 32 per cent., and 62·5 per cent. respectively, were all negative in spite of the fact that the dog flea feeds much more frequently than *P perniciosus* and therefore has a better opportunity of ingesting L.D. bodies from the skin This observation confirms the already abundant evidence that a dog flea is in no way concerned with the transmission of canine Kala Azar. (One flea was found to contain attached and free colonies of *Leptomonas ctenocephali* in hindgut and rectum. These flagellates are readily distinguished from *Leishmania* by their morphology and distribution.) Four wild *Stegomyia fasciata* which fed on dog No. 1 and 30 laboratory bred ones which fed on a heavily infected *Citellus* were all negative. This result is



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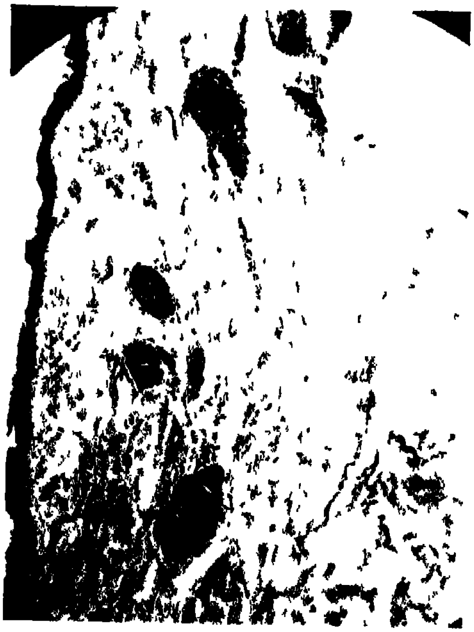

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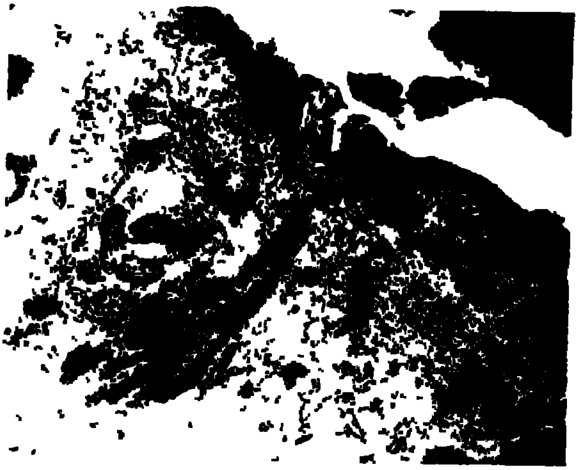

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to be expected, for the intestinal tract of *S. fasciata* usually contains numerous bacteria

Twelve nymphs of *Rhipicephalus sanguineus* which fed on dog No. 2 were dissected after an interval of 6 to 14 days and were found to be negative by microscopic examination.

The significance of the above observations for the transmission of canine visceral Leishmaniasis is obvious A considerable percentage of sandflies, *P. perniciosus*, which feed on naturally infected and in some cases apparently healthy dogs become infected by ingesting skin juices. The visceral infections in the dogs examined were too slight to cause an invasion of the blood stream of sufficient intensity to induce an infection rate in *P. perniciosus* approaching that found in our experiments In infantile Kala Azar the infection of the spleen is far heavier than that found in naturally infected dogs, but the infection rate in *P perniciosus* fed on human cases is negligible as compared to that obtained by feeding on dogs Working with selected cases of infantile Kala Azar in Catania an infection rate of 6 25 per cent was obtained in *P perniciosus*. In Malta, out of 113 *P perniciosus* fed on two cases of infantile Kala Azar, none became infected.

A very slight skin infection is sufficient to produce a considerable infection rate in sandflies. The infectivity of the Maltese canine strain of *Leishmania* for *P perniciosus* is far greater than that of *L tropica* for *P. sergenti* or *P. papatasii* When the infection rate in *P perniciosus* fed on dog No. 1 was about 90 per cent, the number of parasites in the skin was far less than in cases of Oriental sore, which gave a 60 per cent infection rate in *P sergenti* and a 13 per cent infection rate in *P papatasii* (1929)

It is evident that the pathology of canine Kala Azar is very different from that of infantile Kala Azar The visceral infection in dogs is less intense than that of human beings, although the changes in the spleen, liver, glands and bone marrow are of the same type in both species Skin infection is absent or negligible in human Mediterranean Kala Azar as proved by the most delicate test for *L. infantum* in the skin, *i e*, feeding experiments with *P perniciosus* This difference in pathology does not imply that the human and dog strains are different, for the human strain also produces skin infections in *Cricetulus griseus*, *Cricetus auratus*, *Microtus guentheri* and *Citillus citillus* The degree of skin infection varies even in closely related species, being most marked in *Cricetulus griseus* and least in *Cricetus auratus*. In *Cricetulus griseus* the skin infection is established from 2 to 8 weeks after the visceral infection has become intense, while in the dog the skin infection is present even when the visceral infection is very slight. There is no selective infiltration round the hair follicles in *C griseus*

It is important to note that external skin changes have never been observed in dogs infected in the laboratory either with a human or a dog strain Yakimoff (1915), who described the external skin changes in infected dogs both in Tunis and Turkestan, inoculated four healthy dogs with the spleen pulp of naturally infected dogs The animals acquired visceral Leishmaniasis but the skin was normal Donatien and Lestocquard (1929), in a comprehensive review of canine Kala Azar, also remark that the conditions found in naturally infected animals have not been produced in experimental ones This discrepancy may be due to the fact that all the recorded experimental infections of dogs have been produced either by intraperitoneal or intrahepatic inoculations, while in nature dogs are infected via the skin It is obvious that experimental Kala Azar in dogs must be studied *de novo*

There are still important gaps in our knowledge of natural canine Kala Azar Canine Kala Azar is a seasonal disease which shows no important annual fluctuations From a study of the literature it is evident that new cases in considerable numbers appear towards the end of March and in April, *i e*, about the same time that the incidence of infantile Kala Azar is at its height and almost 2 months before the commencement of the sandfly season After September the incidence falls rapidly By the end of November most of the infected animals have either died off or have been cured spontaneously On the basis of the theory of transmission by sandflies the incubation period in the animals found infected in March and April is no less than 5 to 6 months, for *P perniciosus* is very rare after the end of October From the point of view of transmission by *P perniciosus* from dogs to man or other dogs, only the skin infection is important At present there are no data to indicate at what stage of the sandfly season the skin condition in naturally infected dogs is sufficiently advanced to induce an appreciable infection rate in *P perniciosus* Until this point is settled the study of the epidemiology of canine Kala Azar cannot be completed, but even in the present elementary state of knowledge it is clear that from the middle of September to the end of October many sandflies *P. perniciosus* acquire infections with *Leishmania* from the skin of naturally infected dogs

Summary.

Eleven out of 100 dogs examined in Malta between June and November were found to be naturally infected with Kala Azar.

Seborrhea and partial depilation are the most frequent signs of canine Kala Azar and were found in 9 out of 11 cases

The skin condition is due to infiltration of macrophages round hair follicles Infected macrophages are found throughout all parts of the skin both in the areas of infiltration round the hair follicles and in normal dermis

Skin infection may be present in dogs with a slight visceral infection

Histologically the skin infection can be readily differentiated from that produced by *L tropica*

Sandflies, *P perniciosus*, were infected with *Leishmania* by feeding on the unbroken skin of three dogs The infection rates were 62·5 per cent, 65 4 per cent, and 32 per cent respectively

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EXPLANATION OF PLATES.

PLATE 19.

FIGS. 1-3.—Various stages of skin infection of canine Kala Azar.

FIG. 1.—Skin of dog No. 3. Infiltration round a hair follicle. The dermis is normal. (Magn. 120 ×.) Infection rate in *P. perniciosus*, 32 per cent.

Fig. 2—Skin of dog No 2 Several hair follicles are surrounded by infiltrations of macrophages. The intervening dermis is normal. (Magn. 80 ×) Infection rate in *P. perniciosus*, 65 4 per cent.

Fig 3—Skin of dog No 1 Infiltration round hair follicles and sebaceous glands Small areas of infiltration in dermis (Magn. 80 ×) Infection rate in *P. perniciosus* at time skin was taken for section, 90 per cent

Plate 20

Fig 1—Infected macrophages round a sebaceous gland (Magn 820 ×)

Fig 2—Infected macrophages in the neighbourhood of a hair follicle (Magn 820 ×)

Fig 3—Infected macrophages in the normal dermis (Magn 820 ×)

Fig 4.—Single large infected macrophage in normal dermis (Magn 820 ×.) Note absence of tissue reaction in figs 3–4

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Myotatic Reflexes in Sympathectomised Muscle.

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(Communicated by Sir Charles Sherrington, F R S —Received January 21, 1932)

(From the Physiological Laboratory, Oxford)

[Plates 21–23]

Introduction

As a result of their experiments on normal, spinal and decerebrate animals Hunter and Royle (1924) concluded that the post-ganglionic fibres of the sympathetic nervous system constituted the efferent limb of the reflex arc subserving postural contraction in skeletal muscle In a recent article (1931) the present author described a series of experiments on decerebrate cats in which Royle's observation of diminished resistance to passive flexion in a sympathectomised "decerebrate" extensor muscle was confirmed, but the hypothesis that this was due to a "tonic" nervous influence of sympathetic axones on skeletal muscle fibres was denied This denial was based on the fact that postural contraction was modified but not abolished by sympathectomy, and that the operation actually enhanced such proprioceptive reflexes as the shortening reaction and the crossed extensor reflex of Philippson (1905) Finally it was suggested that the diminished resistance of the extended "decerebrate" limb to passive flexion and the enhancement of those proprioceptive reflexes which could be elicited by stretching the sympathectomised muscle were produced by a disturbance of the excitability of its proprioceptive end-organs

Denny-Brown (1928), after studying postural reflexes, has stated that the sympathetic nervous system is not essential to the stretch reflex, but he further stated that he was unable to discover any effect on general posture by excision of the sympathetic

The present author has now repeated his own previous work and has examined the myotatic reflexes of single sympathectomised muscles by means of Sherrington's isometric myograph and fall-table

The naked eye observation of single muscles during the dissection of the limbs of decerebrate cats has thrown further light on the difference in the behaviour of the normal and the sympathectomised muscle Stretching the tendon of a "decerebrate" soleus muscle, within 1 or 2 hours after removal of its sympathetic innervation, produces a strong postural contraction which closely resembles the response elicited by the same stimulus from the normal muscle If the stretching force be moderate the contraction appears to be equally well maintained in both muscles, if, however, the applied tension be increased, the resistance of the sympathectomised muscle yields first, and the whole muscle extends under the influence of the lengthening reaction

This liability to undergo reflex lengthening is increased as the interval of time following sympathectomy increases up to a period of at least 3 weeks In the "chronically sympathectomised" muscle (from 10 to 80 days following operation) the gradual application of tension appears to produce a series of "fractional" lengthening reactions, in which slight early resistance and partial relaxation follow one another in rotation, until considerable tension is developed by passive stretch of the muscle elements. If the muscle is stretched sharply, the response is not unlike that of a flexor muscle, there is at first a strong "pluck" reflex, followed by a lengthening reaction, and at this new length the muscle may exhibit a weak postural contraction

Sympathectomised muscle displays a marked tendency to exhibit clonic movements, both in acute and chronic preparations Clonus, usually a prominent feature of intercollicular decerebrate preparations manifesting good rigidity, is profoundly influenced by tonic vestibular and neck reflexes, so that for comparative purposes a symmetrical disposition of the animal is essential. Under such conditions, one may say that the sympathectomised muscle is always, as it were, on the verge of substituting clonus for postural contraction Any unevenness in the rate of stretch is sufficient to bring about rapid clonic movements, of very slight tension, in the sympathectomised muscle The chief "clinical" differences to be observed, between clonus on the normal and sympathectomised sides, are that, in the latter, the rate appears to be more rapid and the clonic movements occur over a wider range of tension. In the normal muscle, clonus, which occurs over an optimal range of tension, may be arrested by the application of further stretch to the tendon In the sympathectomised muscle such further stretch may arrest clonus for several beats, during which time the muscle may be felt to lengthen, with the subsequent resumption of clonus at this new length The profound influence of the tonic neck and vestibular reflexes, in producing a subliminal excitatory state at the central motoneurone pool, is well demonstrated when, with the head and neck in the "maximal" position, the freely dissected sympathectomised muscle, lying slack on its surrounding muscular bed, may be seen to be in clonus Such atonic clonic movements, which have not been observed in the normal muscle, can only be produced by the centripetal excitation, resultant on sympathectomy coinciding with the subliminal excitatory state already existent in the motoneurone pool That this is so is shown by cessation of clonus where the head and neck are moved to a less maximal position

Further evidence, of peripheral excitation summing with a subliminal central excitatory state, has been seen in several decerebrate cats in which rigidity was not marked When these animals were disposed symmetrically with the head and neck in the "maximal" position the sympathectomised limb might be seen to exhibit a partially extended attitude while the normal limb remained flexed This asymmetry, which has been noted before (Phillips, 1931), depends upon a stronger postural contraction in sympathectomised muscles, produced almost certainly by increased proprioceptive excitation; it being assumed that a constant inequality in vestibular or cutaneous influence is most unlikely over a series of animals

From clinical evidence it may be said that sympathectomised muscle retains in great degree its power to respond to a stretching force by contracting, loses in great degree its ability to maintain a postural contraction, and in place of the latter displays a tendency to replace tonus by clonus The subsequent myographic analysis substantiates and amplifies this summary statement.

Technique.

A double mirror type myograph as described by Sherrington (1928) and Eccles and Sherrington (1929) has been used in making simultaneous records from two corresponding muscles. The twin torsion wires of this myograph were either 94/1000 or 64/1000 inch in diameter. The former pair were used for examining stretch reflexes, the latter for muscle jerks and records of clonus. Both pairs of wires were calibrated and proved to record equal deflections for equal loads. A modification was made in the original design of the myograph, a third mirror being attached just below and between the twin torsion wire mirrors This could be rotated into a suitable position before attaching the muscles to the myograph and then fixed by a small binding screw so that it recorded a base line of zero tension to both torsion wires An optical recording system was used, in which the image of an illuminated slit was reflected by the three myograph mirrors on to a falling plate camera.

Graded stretch stimulation was provided by means of a Sherrington pattern fall-table (1924)

The sympathetic chains have been removed from 35 cats through a midline incision in the anterior abdominal wall, the whole extent of each chain being excised from the crus of the diaphragm above, down to and including the second sacral ganglion below In 25 animals the left chain was removed, in 8 the right chain, and in 2 cats both chains were excised Twelve of these cats were decerebrated and examined immediately after the sympathectomy, while 23 were allowed to recover from the operation and were decerebrated and examined from 10 to 80 days later In the cat the midline operation offers the best approach to the abdominal sympathetic chains, since it permits a clear view of both of them One chain may be removed *en bloc* by careful blunt dissection without disturbing the relations of its fellow of the opposite side. Post-mortem examination always confirmed the integrity of the undisturbed chain on the control side Protected by the sheath of extraperitoneal fat in which it lies, it was never seen to be involved in the peritoneal thickening and mild fibrosis succeeding the operation

The soleus muscles of decerebrate cats have been shown to be the seat of a strong stretch reflex (Denny-Brown, 1928), their fibres are relatively parallel and their almost linear origin permits a widespread separation of the muscle belly from the surrounding soft tissues. By virtue of those qualities they were selected as presenting a suitable bilateral preparation for the comparative study of postural reflexes in homologous muscles They were prepared for the myograph by free dissection of the muscular bellies from the surrounding soft tissues At the site of their insertions a small piece of bone was chipped off the calcaneum and left attached to the tendon. All other muscles in both hind limbs were paralysed by motor nerve section, and the cutaneous nerves were divided according to the technique described by Sherrington (1909) The decerebrate animal was placed prone on the fall-table with the trunk directed at right angles away from the myograph and supported on each side by warm bottles The head was extended and maintained erect in the midline by attaching to an upright stand a loop of the silk used for suturing the scalp wound The fore limbs were extended side by side on the table surface The hind limbs were fixed to the fall-table in the same vertical plane by means of drills inserted transversely through the upper and lower ends of the tibiæ. With the origins of the soleus muscles fixed in this manner their respective tendons were attached to the horizontal tension bars of the torsion wires by means of hooks of adjustable length. These hooks, constructed like small union screws, an insulating fibre cylinder forming the union, permitted a separate adjustment of the length or tension in each muscle When the table was allowed to fall through a known distance the two soleus muscles linking it to the rigid myograph were submitted to the same stretching force

The electric responses were led away from each muscle by silver chloride-coated electrodes to a double string galvanometer Shadows of the two galvanometer strings were projected on to the slit of the falling-plate camera The silver chloride electrodes were inserted in corresponding regions of the two muscles approximately to the same depth and at the same distance apart The tension in the galvanometer strings was adjusted before each plate-record, so that a potential difference of 1 millivolt produced a deflection of 15 mm in each string

Myotatic Reflexes

The quality of plasticity exhibited by "decerebrate" muscle demands a low initial tension in making simultaneous comparative records of stretch reflexes in two muscles The term "plasticity," originally applied by Sherrington (1909), expresses the ability of the muscle to present the same tension at different lengths This quality, then, when two "decerebrate" muscles are submitted to the same tension, may result in unequal length, an asymmetry which carries in its train an unequal development of tension on the application of further stretch It is important therefore to ensure the same initial length in the two muscles before they are stretched This was accomplished as follows The two union-screw hooks were suspended on their respective tension bars and the three myograph mirrors rotated so that the beams of light reflected by them were focussed at one point on the camera slit, this point being that of zero tension in both torsion wires The fall-table was then raised and each soleus tendon impaled on its hook, both muscles being quite slack The hooks were then shortened separately, taking up the slack in the muscles until the first minimal deviation of the mirror beams, away from the base line of zero, could be observed Under such conditions each muscle was subjected to an initial tension of less than 5 grams, and, as can be seen in the records, the galvanometer strings were "silent," or exhibited activity in only one or less frequently two muscle fibre units, before the onset of the table fall. This bilateral minimal posture has been taken as the starting point for the comparison of the responses of the normal and sympathectomised muscles to stretch.

Control myograms in five animals, either with both sympathetic chains intact or both excised, have shown, in records from both soleus muscles, the simultaneous development of tension at the same rate with the achievement of approximately the same final plateau tension, provided that the amount of stretch was small, the rate of stretch constant and the initial tension low If these conditions were satisfied, the muscles under a stretch of 3 mm carried out at an even rate presented a strong myotatic reflex over 90 per cent. of which was constituted by active contraction If the amount of stretch was increased beyond 6 mm , the passive tension produced by stretch of muscle elements claimed a much larger percentage of the total tension, fig 10, Plate 22 The electric responses set up by the table fall in the soleus muscles of normal or bilaterally sympathectomised cats always appear within 5 sigma of each other, figs 1 and 2, Plate 21 The latent periods of the myotatic reflexes in the muscles of bilaterally similar preparations can be said, therefore, to vary under the present technique by less than 5 sigma

Myograms taken from decerebrate cats, in which the sympathetic chain has been removed on one side, display characteristic differences on the two sides, both in the mechanical and the electrical records It is worthy of note that these differences were not due to asymmetrical decerebration, since they were constantly present in 35 animals Intercollicular decerebration by unilateral subtemporal craniotomy, the operation performed in each case, has been shown by post-mortem examination to permit a macroscopically symmetrical brain-stem section That the changes were not produced by centripetal impulses streaming in from skin wounds has been proved by dusting these with an anæsthetic powder

The sympathectomised muscle attains for the same stretch a greater myotatic tension, fig 3A, Plate 21, and its mechanical record arises more sharply from the base line, fig 3, Plate 21 In an acutely sympathectomised muscle a final plateau of moderate tension is fairly well maintained in some cases This contrasts with the behaviour of chronically sympathectomised muscle, which does not maintain even a moderate plateau tension, the mechanical record falling, in contrast with the normal side, to a lower level, fig. 5, Plate 22, on the one hand, or, on the other, passing into clonus, fig. 8, Plate 22 In chronic preparations the tension ascent from the base line follows a series of hump-like contractions, fig 5, contrasting with the tolerably smooth ascent of the tension curve of the normal muscle This uneven rise is due to synchronous contraction in several muscle fibre units, a form of activity which, as will be shown later, is reflected in the galvanometer record.

The electric responses at once point to a much shorter latent period of the stretch reflex on the sympathectomised side. In fig. 4, Plate 21, where, in the minimal posture described above, a single unit is firing in each string before the application of stretch, the electric responses commence some 50 sigma earlier on the sympathectomised side after the onset of the table fall. This earlier response, also seen in fig 3A, Plate 21, may be taken as an expression of the fact that the centripetal impulses flowing in from the two muscles produce a central excitatory state which first becomes liminal in the motoneurone pool of the sympathectomised muscle This earlier achievement of centrifugal firing, under identical external conditions of peripheral stimulation, may be due either to the activity of more proprioceptive end organs, or to the enhanced activity of the same number, or to both factors occurring together In normal muscle, both during the application of stretch and during the maintenance of postural contraction, unless it be of that slight order produced by the activity of but a few units, there is to be found a characteristic and complete asynchronism of firing (Fulton and Liddell, 1925), seen in the electrical record as a restless series of small deflections This asynchronism, produced by the application of stretch at an even rate, is responsible for the steady rise of the mechanical record In sympathectomised muscle, both during the application of stretch at an even rate and following the achievement of the final plateau, there can be seen to be many short "silent periods" in the activity of the galvanometer string They produce the uneven ascent in the mechanical record, where they may occur simultaneously with an increase in mechanical tension, the latter representing the after-action of the preceding contraction shown by the large compound action currents, fig 2, Plate 21 These "silent periods" therefore do not represent the lengthening reaction, although they must be due to the temporary predominance of proprioceptive inhibition, producing, in conflict with proprioceptive excitation, a transient central inhibitory state in the motoneurone pool The descent of the plateau tension, the contraction being isometric and associated with short periods of subliminal central excitation, is explained by the fact that the falling tide of central excitation at the onset of each "silent period" is no longer reinforced by the rising tide of peripheral excitation, associated with increasing stretch during the table fall.

These "silent periods" are no doubt the basis of the clinical phenomena in chronic sympathectomised muscle which earlier in this paper have been termed "fractional" lengthening reactions They are present, but less obvious, in acute sympathectomised muscle, fig 3A, Plate 21 That the influence of sympathectomy has not been to bring proprioceptive inhibition alone into a prepotent condition is shown both by the greater tension achieved by sympathectomised muscle during stretch and by the characters of the action currents produced by such contraction

The electric responses from sympathectomised muscle always include large compound waves diphasic in type which are not characteristic accompaniments of evenly applied stretch to normal muscle, fig 3A, Plate 21 These waves are evidence of the synchronous or almost synchronous activity of several muscle fibre units, that is of two or more cells of the motoneurone pool This is interpreted as being due to the simultaneous activity of several proprioceptive end organs in their firing into the motoneurone pool which is a parallel with the mechanism suggested to be responsible for the shorter latent period of the stretch reflex in sympathectomised muscle It is the simultaneous activity of several centripetal afferents which is emphasised rather than the general intensification of their individual activity

Denny-Brown has demonstrated the profound influence of proprioceptive inhibition on the motoneurone pool not only of the excited muscle but also of other muscles (1928)

The intensity of such inhibition is largely conditioned by the extensity of synchronous firing in muscle fibre units In normal muscle with the galvanometer string record as an index, proprioceptive inhibition achieves a maximum in the synchronous contraction type, the muscle jerk, where the length of the "silent period" is proportional to the height of the jerk contraction and is seen to be at a minimum in the completely asynchronous firing of steady maintained postural contraction Sympathectomised muscle is believed to achieve an intermediate position of incomplete synchronisation in which asynchronous characteristics such as steady posture are in great degree lost, while, as will be shown later, synchronous activity such as clonus and the muscle jerk are in great degree enhanced

The thesis set down above to explain the fall of postural tension shown by the decline of the mechanical record following stretch suggests in the light of the alternating central excitation and inhibition manifested in the electric responses from sympathectomised muscle that the rate of stretch may be a critical factor in determining the maximum tension achieved by the resultant myotatic contraction That such is not the case in normal muscle may be seen from the original records published by Liddell and Sherrington (1924) where a slow stretch produced an even greater maximum tension than the same stretch carried out at a very fast rate

The records they publish, however, were produced by stretches of 8 mm. with a resultant passive tension of more than 35 per cent. of the total. With this factor eliminated it may be seen that the same stretch applied either slowly or rapidly to normal "decerebrate" muscle elicits within limits the same maximal tension On the other hand, this does not occur with sympathectomised muscle, which under the slow stretch fails to achieve as great a tension as under the fast, figs 12 and 13, Plate 23 There is therefore a critical rate of stretch at which the normal and sympathectomised muscle should achieve the same maximum tension, a rate which is very closely represented in fig 5, Plate 22 With rates faster than this the sympathectomised muscle contraction is greater than the normal myotatic contraction and with slower rates it is less

All these foregoing observations are regarded as an expression of the alternating synchronous activity and "silence" in the galvanometer string record from the sympathectomised muscle They are considered to be based upon the quantitative value of the rising tide of peripheral excitation produced by graded rates of stretch occurring during the critical "silent period" in the string record If this excitation be small there is but slight reinforcement of the mechanical tension, if it be large the contraction is strongly reinforced The absence of such "silent periods" with complete asynchronous firing in the normal muscle removes this factor determining the maximum tension to be achieved

That there is little or no change in the activity of proprioceptive end organs of low threshold following sympathectomy is proved by examining the rate of firing of single muscle fibre units under minimal conditions of applied tension. It might be expected that if the excitability of low threshold receptors was increased by sympathectomy, the rate of firing of single muscle fibre units would be faster in the sympathectomised muscle than in the normal when the same minimal stretch was applied to both muscles To examine this the decerebrate cat was attached and suspended in the prone position to a horizontal steel bar by means of vertebral clamps With the animal's body in a symmetrical position the freely dissected soleus muscles depended from their bony origins alone The muscle electrodes were inserted as before and correspondingly small weights were attached to each tendon When a 2 gram weight was suspended from each soleus it was found in many instances that a single unit was caused to fire in each muscle, fig 6, Plate 22 Both on the normal and sympathectomised sides the rate of firing was found to correspond almost exactly and to vary from 9 to 11 per second. Such minimal stretch was seen therefore from the nature of the centrifugal firing to produce identical streams of centripetal excitation from each muscle. If, however, the 2 grams were substituted on each side by a 10 gram weight a great difference in the electric responses was at once evident, fig. 7, Plate 22 The added stretch was usually only sufficient to bring into action two or three more units Owing to asynchronous firing each unit was capable, by analysis, of isolation from its fellows On the sympathectomised side, however, the same stimulus produced firing in too many units to permit their number to be determined The string record was characterised by the presence of large compound waves, diphasic in type, alternating with periods of "silence," indicating a state of partial synchronism bordering on clonus It seems justifiable to conclude from this latter observation, in the light of the local nature of stretch excitation, that a greater number of proprioceptive end organs were in simultaneous activity in the sympathectomised muscle and were producing an almost synchronous firing in several motor nerve cells The influence of sympathectomy was, therefore, to produce a greater concentration of tension receptor activity at low threshold by activating end organs, which in the normal muscle could only be brought into play by the application of further stretch

Muscle Jerks and Clonus

The soleus jerks have been compared by means of an electromagnetically released myograph. This instrument, fig 1, for whose construction I am indebted to Mr O'Neill of this laboratory, consists essentially of the three mirror myograph outlined above with torsion wires of 64/1000 inch diameter Small brass blocks are mounted on the ends of the horizontal tension bars of the torsion wires and through these blocks iron screws are inserted with their points upward and their flat circular heads downward. Mounted below the solid brass base of the myograph, a stage projects carrying two single pole

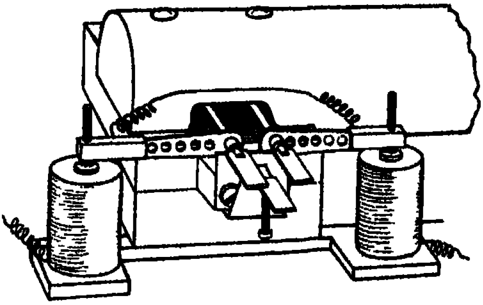


FIG 1.—Diagram of the electromagnetically released triple mirror myograph used for recording soleus jerks

electromagnets, the soft iron core of each of which is so placed that it lies immediately below the circular head of one of the iron screws. When a current at a potential of 6 volts is passed in series through the two electromagnets the screws are pulled down on to the soft iron cores, and with the magnification provided by the optical recording system a considerable deflection of the mirror beams is produced at the slit of the camera. The height of each iron screw is then adjusted so that the extent of this deflection is the same for the two beams At the instant of breaking the circuit in the magnet coils the screws are released from the iron cores and the unbalanced torque in the torsion wires produces an immediate return of the mirror beams to and beyond the zero base line about which they vibrate with the natural period of the wires before coming to rest.

This instrument provides a means of applying the same instantaneous stretch to two muscles, thereby producing comparable records of muscle jerks To examine these the decerebrate animal was arranged in the position described for the myotatic reflex with the two soleus muscles attached to the myograph under identical conditions of minimal stretch. The fall table was then lowered just enough to cause a 5 mm deflection of the torsion-wire beams away from the zero base line at the camera slit As the screws of the electromagnetic release were so adjusted that on closing the circuit equal deflections of at least 40 mm were produced in each beam the two soleus muscles were then subjected to identical conditions of minimal stretch posture On breaking the circuit both muscles were submitted to the same slight instantaneous stretch with the production of typical muscle-jerks Control observations using two normal " decerebrate " muscles showed corresponding jerks in both, as judged by the amount of tension developed, the characters of the action current, and the length of the " silent period " Sympathectomised muscle, however, always presented under these conditions a jerk of greater tension, accompanied by a larger action current wave and a longer " silent period " than in the normal muscle, figs 8 and 11, Plate 22 Following the jerk sympathectomised muscle often relapsed into a condition of incomplete clonus, fig 8, Plate 22 Soleus jerks produced by tapping the table with a rubber covered rod always elicited a contraction of greater magnitude on the sympathectomised side, fig 9, Plate 22. To the isotonic nature of the contraction and the weight of the leg is due the probable explanation of the failure to detect a larger amplitude of the knee jerk in the sympathectomised limb when the decerebrate animal is examined clinically.

It has been found difficult to obtain bilateral records of clonic movements due no doubt to interference in the corresponding " half-centres " of the cord.

The records which have been obtained, however, show in accordance with the clinical observations made above that in sympathectomised muscle clonus is more prone to occur, occurs at a much faster rate and may be manifested at all postural tensions of which the muscle is capable, it being remembered that steady posture of other than moderate tension is not seen in sympathectomised muscle Fig 14, Plate 23, shows a record of bilateral clonus, in which the low tension and rapid clonic rate has produced a wavering mechanical record rather than a true clonic record From the electrical records, however, it may be seen that while the normal muscle beats at a rate of 15 per second, with definite synchronous action current waves alternating with periods of complete or almost complete string "silence," the sympathectomised muscle on the other hand is beating so quickly that the rate is difficult to determine, the action current waves are very small and are so close together that the "silent periods" are almost abolished The rate of clonus depends fundamentally on the brevity of the "silent periods" The concentration of receptors at low threshold provides a rapidly rising tide of peripheral excitation early in the onset of clonic relaxation, determined by the central inhibitory state, which is itself produced by the preceding synchronous firing It is believed that sympathectomy influences clonus in this way in that the synchronous firing of receptors at low threshold sets up a wave of centripetal impulses which abolishes the "silent period" immediately after its onset, and replaces it by a weak central excitatory state in a few cells of the motoneurone pool. In this way there is produced centrally a rapid alternation of just supra-liminal and just sub-liminal excitation which is manifested peripherally as a rapid clonus of low tension

Whenever the proprioceptive end organs of sympathectomised muscle are used to excite contraction either in the muscle itself or in other muscles the characters of such contraction, as judged by the galvanometer string record, are those that have already been noted. Traction on the tendon of one soleus sufficiently strong to produce a lengthening reaction in it excites contraction in the opposite soleus This is a similar reflex to that described by Philippson (1905) for the quadriceps femoris A contraction of greater tension is produced in the reacting soleus when the sympathectomised muscle is used to excite it than when the normal muscle is employed, fig. 16, Plate 23 And yet it may be noted that soleus contraction produced by faradic stimulation of a contralateral muscle nerve is equal in both the normal and sympathectomised muscles.

It is *the inclusion of the receptor end organs in the reflex arc* that produces the observed changes in contraction tension following sympathectomy. In this Philippson type-reflex the nature of the centrifugal excitation sampled by the reacting soleus may be observed in the galvanometer string record When the sympathectomised soleus is used as the exciting muscle the string record from the reacting muscle displays the characteristic large compound waves and short periods of "string silence" described before With the normal soleus as the exciting muscle the string record displays completely asynchronous activity, with little or no sign of transient central inhibitory state, fig 16.

This is regarded as evidence of *concentration* of receptor activity at low threshold in sympathectomised muscle

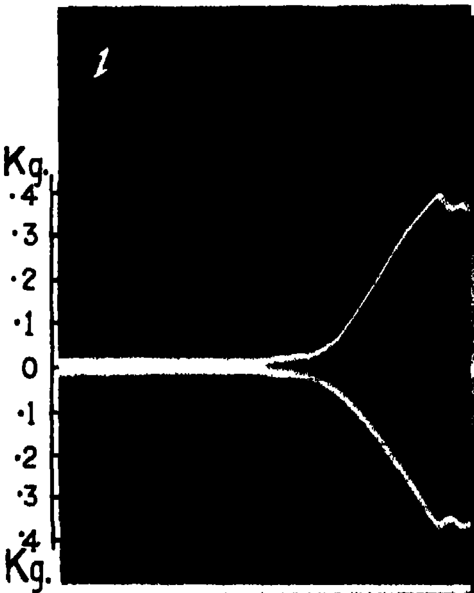
Discussion

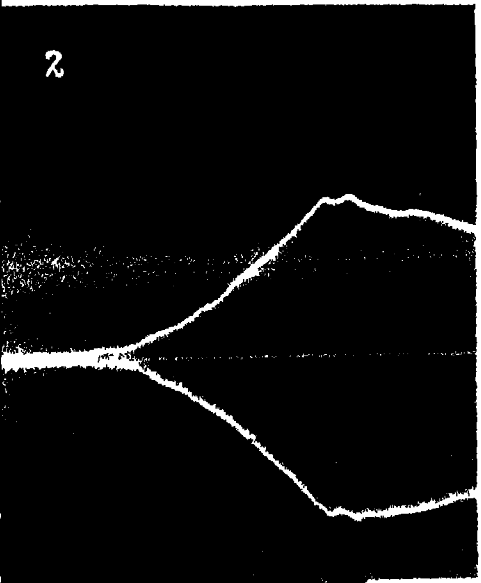
It is felt that the analysis of changes of receptor threshold in sympathectomised muscle presents a formidable problem, but nevertheless it is believed that a satisfactory working hypothesis may be constructed from the foregoing evidence It is at the same time important to state, in agreement with Denny-Brown, that there is no evidence that the sympathetic innervation to skeletal muscle plays any qualitative part in postural reactions, and that changes in proprioceptive reflex action (Royle, 1924) which have been thought to do so may be explained on a basis other than the sympathetic motor innervation of muscle fibres.

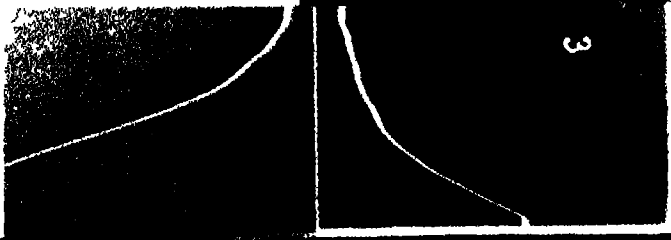
A significant observation is the progressive change in reflex action following sympathectomy, the loss of steady postural contraction and the tendency to display clonic movements becoming most prominent some weeks after the operation Without predicting the results of another investigation which is being prosecuted at the present time it may be said that there is some evidence pointing to a considerable change in the vascular pattern of chronic sympathectomised muscle

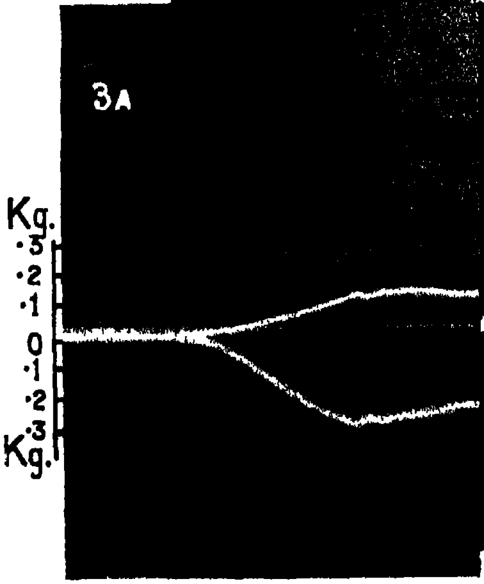
Analysis of the lengthening reactions in normal and sympathectomised muscle throws some light on the behaviour of proprioceptive end organs under graded threshold of stretch This reaction, originally described by Sherrington, is that "melting" of resistance and passive extension of a "decerebrate" muscle when an increasing tension is applied to its tendon

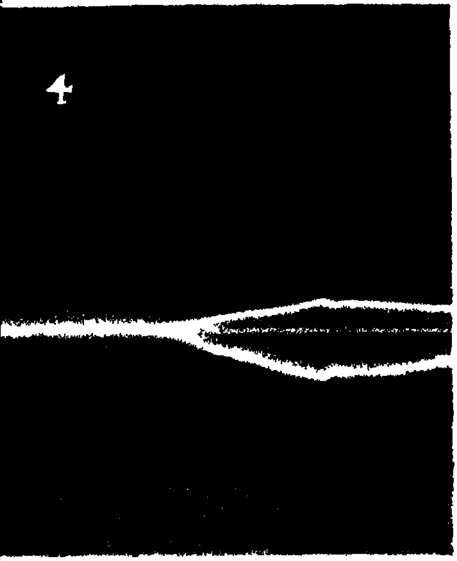
It may be said to constitute the clinical basis for the estimation of so-called "tonus" in skeletal muscle, and, as has been stated above, is produced at a lower threshold of applied tension in sympathectomised muscle. It is a reflex action in so far that it depends on the activity of end organs, the stimulation of which produces centripetal impulses that lower the central excitatory state. That the lengthening reaction does not involve the abolition of central excita-











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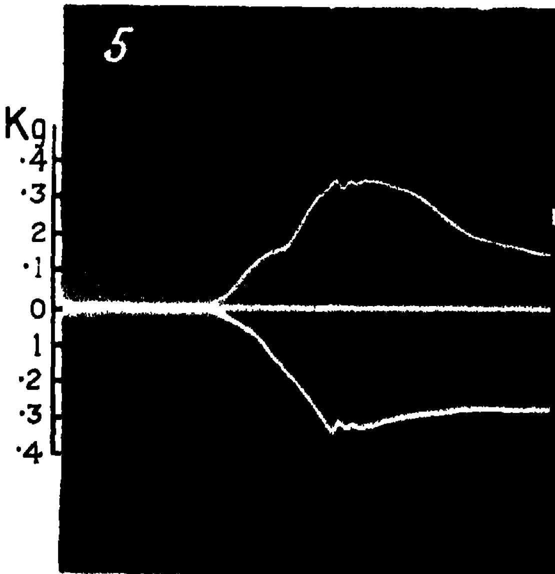

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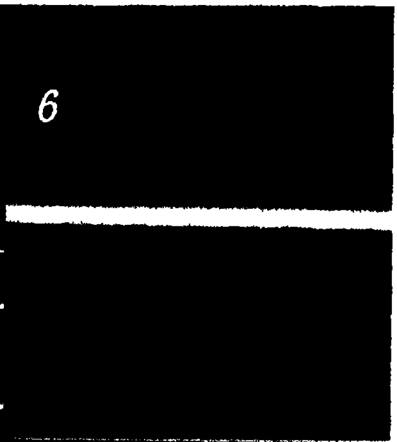

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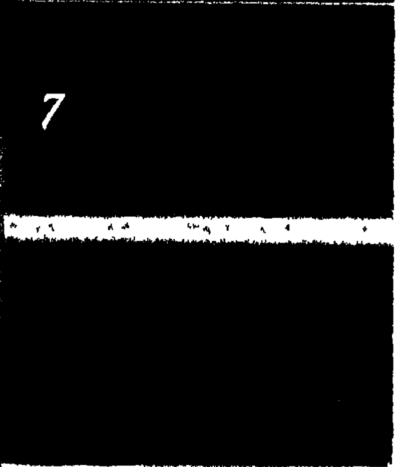

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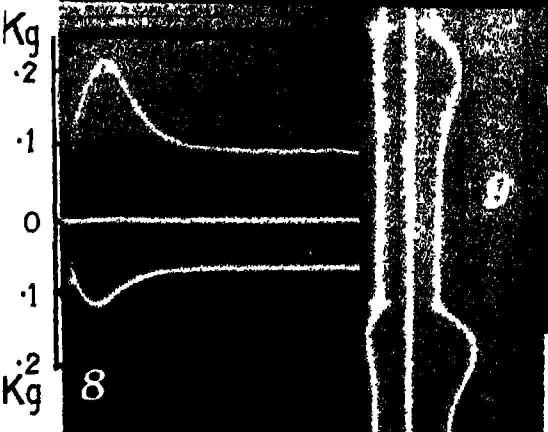

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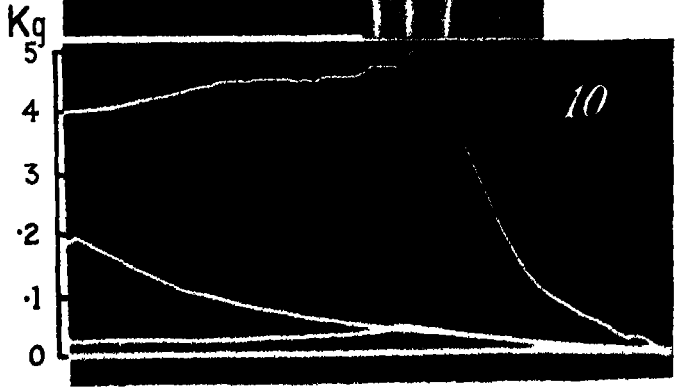

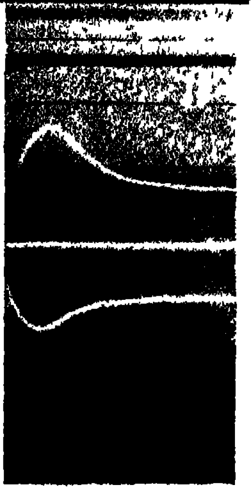

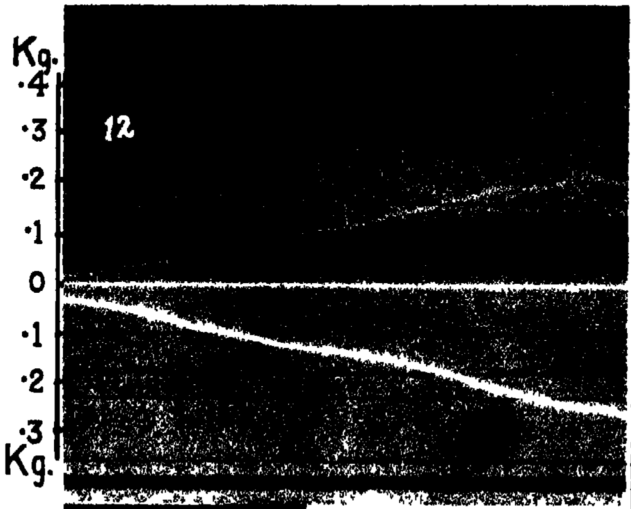
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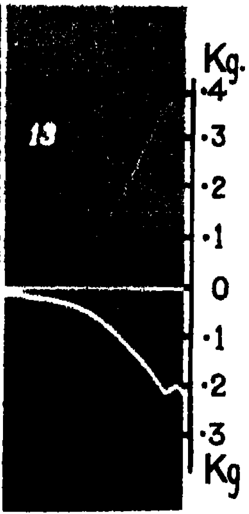
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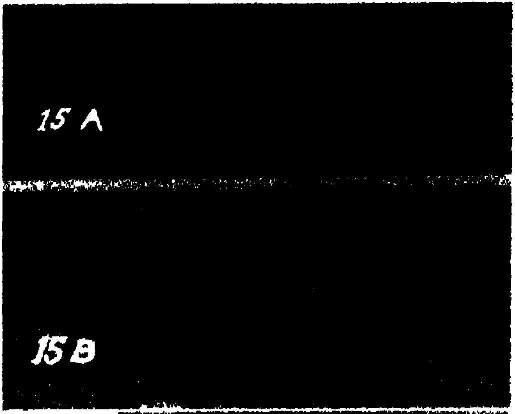
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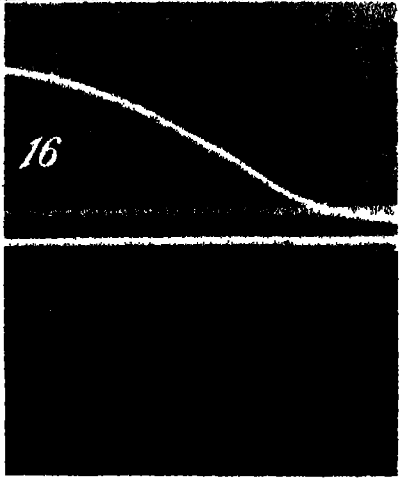
15 B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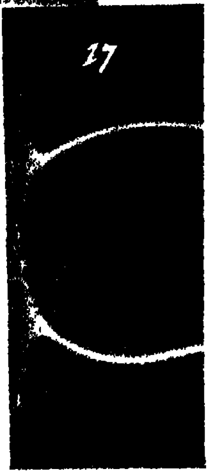
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tion with the consequent production of a state of temporary central inhibition may be seen in fig 15, Plate 23. A "silent period" in the galvanometer string record is neither an index nor a characteristic accompaniment of the lengthening reaction Both in normal and in sympathectomised muscle string activity is a constant concomitant of the lengthening reaction It is early in the application of tension that a difference is to be noted in the electric responses from normal and sympathectomised muscle In the normal muscle complete asynchronous activity of the string record is seen throughout the application of tension both at low and at high threshold This is a characteristic feature of myotatic contraction in normal muscle subjected to evenly increased stretch unless the rate of stretch is sufficiently great to elicit a muscle jerk In sympathectomised muscle this asynchronous activity is seen only at high threshold of applied tension, fig 15, Plate 23 At low threshold, on the other hand, there is to be seen the combination of large compound waves and short "silent periods" which have already been taken as evidence of incompletely synchronised firing in two or more muscle fibre units This is no doubt due to the simultaneous activity of low threshold receptors in their firing into the motoneurone pool The asynchronous activity of normal muscle suggests the presence in it of a group of stretch-excitor end organs which are evenly distributed over a stimulation threshold gradient The low threshold receptors are so excitable that they respond to minimal amounts of stretch although they produce firing at the slow rate of 9 to 11 per second It has been observed that in sympathectomised muscle these low threshold receptors still produce firing at the same rate, fig 6, Plate 22, but that at a slightly higher threshold a group of receptors are brought into activity together

On such evidence it is submitted that sympathectomy has produced a disturbance of the even distribution of stretch-excitor end organs over a threshold gradient in such a way as to produce, by increasing the excitability of a number of receptors to the same or almost the same level, a concentration at low threshold of end organs having the same or almost the same excitability. Such an hypothesis, which it is attempted to represent diagrammatically in fig 2, serves to explain all the changes in proprioceptive reflex activity which have been described to follow the removal of the sympathetic innervation.

Concluding Remarks

The changes in postural reflex activity following the removal of the sympathetic innervation to skeletal muscle in both normal and pathological states may now be considered briefly.

In the normal animal no change in posture and proprioceptive reflex activity is seen The loss of maintenance of postural contraction which has been shown to be present in chronic sympathectomised muscle is in the normal animal most probably compensated by the striking of a new balance in the fractions of the body weight supported by the myotatic contraction in the extensor muscles of the limbs of opposite sides The difference in the amplitude of the knee jerks is masked by the isotonic nature of the contraction and particularly in large animals by the inertia of the limb. In the normal animal with intact extero- and proprioceptors, which, during examination of the animal, contribute an irregular stream of conflicting centripetal impulses to the motoneurone pool, a rapidly alternating state of just supraliminal and just subliminal excitation, shown to be characteristic of clonus in sympathectomised muscle, is prevented

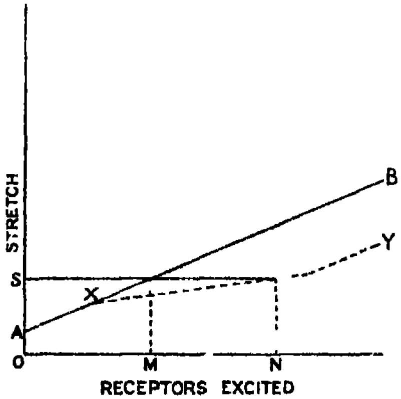

FIG 2 —A × B — normal threshold gradient of receptors exciting myotatic contraction in soleus muscle A × Y = threshold gradient following sympathectomy A stretch " OS " which in normal muscle activates " OM " exciting receptors, activates " ON " such receptors all at low threshold in sympathectomised muscle

It will be remembered that the clinical changes following sympathectomy, described by the author, have been observed after intercollicular decerebration By thus removing supracollicular inhibition and by the symmetrical disposition of the animal during its examination an attempt has been made to produce in the motoneurone pools of the extensor muscles a relatively constant subliminal excitatory state. Such a condition has permitted the detection of slight constant differences in the quantitative value of centripetal streams of proprioceptive impulses

It is believed that in man also, in pathological states producing a complete or partial removal of supracollicular inhibition and associated with an intact vestibulo-spinal mechanism, slight constant changes in postural activity may be detected following sympathectomy On the basis of the hypothesis suggested above, one may expect an earlier disappearance of extensor spasm in the sympathectomised limb when it is forcibly flexed, due to the earlier onset of the lengthening reaction No change in the amplitude of the spastic knee jerk would be anticipated, but following sympathectomy one would expect the spastic limb to fall into partial flexion following the phasic response of the knee jerk and not to remain in the partially extended attitude characteristic of it before the operation Such a fall is due to the production of the lengthening reaction in the spastic sympathectomised extensor by the kinetic stretch applied to it by the weight of the heavy leg falling immediately after the upward fling of the knee jerk For the reasons given before clonic movements would not be expected to be any more conspicuous after sympathectomy than before it

In conclusion it is suggested that the term "tonus," whose meaning has become so gross, should not be used and that the results of the clinical examination of resistance in spastic extensor muscles should be stated in terms of that proprioceptive reflex, the lengthening reaction

Summary

Changes in the proprioceptive reflex activity of skeletal muscle follow the removal of its sympathetic innervation These changes are progressive and are most prominent some weeks after operation

The myotatic tension produced in sympathectomised muscle by kinetic stretch may be modified by varying the rate of application of stretch, there being a critical rate at which the same amount of stretch elicits the same myotatic tension in both normal and sympathectomised muscle, at rates greater than this the sympathectomised muscle attains the greater tension and *vice versa*

Under static stretch chronic sympathectomised muscle is unable to maintain a postural contraction of other than low tension.

Chronic sympathectomised muscle displays a marked tendency to exhibit rapid clonic movements of low tension at all grades of postural tension of which it is capable

The muscle jerk following sympathectomy displays a greater mechanical tension, a larger action current wave and a longer "silent period."

It is submitted that these changes may be explained by a concentration at low threshold of the activity of those proprioceptive end organs which excite

myotatic contraction Such an hypothesis differs considerably from the view expressed in a previous publication (Phillips, 1931) in that it is now believed that proprioceptive inhibition is conditioned by the nature of the centrifugal firing rather than by the amount of applied stretch

Finally it is concluded that in postural contraction sympathetic axones have no direct efferent influence on skeletal muscle fibres and that the progressively cumulative changes which succeed sympathectomy depend on some trophic alteration modifying the responses of proprioceptive end organs

Acknowledgments

I am deeply indebted to Sir Charles Sherrington for his invaluable advice, for the laboratory facilities placed at my disposal and for his criticism of this paper My thanks are due to Dr H M Carleton for the expert assistance rendered by him at aseptic operations

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DESCRIPTION OF PLATES

All figures read from left to right except figs 10 and 16, which read in the reverse direction. Unless otherwise stated, time is indicated by a tuning fork vibrating at 100 d.v. per second. The myograph records appear as bright lines, the central line when present indicating the base line of zero tension Tension in the torsion wires is indicated by deflection of the mirror beams away from the base line

The table fall is indicated by a descending black line at the top of the figure

All measurements were made from the original plates.

PLATE 21

FIG 1—Bilateral soleus preparation, sympathetic innervation intact on both sides, single muscle fibre unit firing in each soleus before onset of stretch, both firing at rate of 10 per second, table fall = 3 mm in 0 25 second Note tension ascent is smooth and at same rate on both sides with attainment of approximately the same final plateau tensions The electric responses commence 5 sigma late on left side (below), they are completely asynchronous on both sides and of the same order of magnitude

FIG 2—Bilateral soleus preparation Both sympathetics excised, both strings "silent" before table fall of 3 mm in 0 23 second Note unsteady rise at same rate on both sides *to same maximum tension with subsequent fall of both plateau tensions* (Sympathectomy 62 days previously), tension scale as in fig 1 The electric responses commence 4 sigma late on the left side (below) Note short "silent periods" in both strings

FIG 3—Bilateral soleus preparation, normal soleus records on the same side as the time *marker which is a 10* d v per second fork *Left soleus sympathectomised 52 days* previously, table fall = 4 mm in 0 2 second, tension scale as in fig 10

FIG 3A—Bilateral preparation, faulty fixation of base line mirror which is deflected 2 mm toward normal side, normal soleus records on same side as the fall indicator, acute left sympathectomy 3 hours previously, table fall = 3 mm in 0 2 second Electric responses commence 48 sigma late on normal side Note greater maximum tension on sympathectomised side only moderately well maintained Note short "silent periods" in sympathectomised muscle during application of stretch

FIG 4—Bilateral preparation, normal soleus records on same side as the fall indicator, acute left sympathectomy 2 hours previously, table fall = 2 mm in 0 12 second Electric responses appear 56 sigma late on normal side.

PLATE 22

FIG 5—Bilateral preparation, right soleus, sympathectomised 25 days previously, records on the same side as the fall indicator, table fall = 3 mm in 0 26 second.

FIG 6.—Bilateral preparation, left soleus (lower string record) sympathectomised 15 days previously, single muscle fibre unit fires in each muscle at rate of 11 per second under the stretch provided by 2 *gm* weight suspended from each tendon

FIG 7—Same preparation as in fig 6, record of firing set up by replacing 2 gm weights with 10 gm on each tendon

FIG 8—Bilateral preparation, soleus jerks elicited by electromagnetically released myograph, right soleus (recording above) sympathectomised 23 days previously, "silent periods"—*on normal side* 0 12 *second, on operated side* 0 18 second Note incomplete clonus following jerk in the sympathectomised muscle

FIG 9—Bilateral preparation, soleus jerks produced by lightly tapping the fall table with a rubber-covered brass rod Sympathectomised soleus records on same side as the tuning fork, two jerks shown were elicited by tapping the table at widely separated points, tension scale *as in fig* 8

FIG 10.—Single soleus preparation, highest record produced by a table fall of 4 mm in 0 2 second. Lowest record indicates passive tension developed by the same fall at the same rate after section of the motor nerve (8 per cent. of the total), intermediate record shows passive tension developed by same paralysed soleus if the stretch be increased to 7 mm at the same rate (36 per cent. of the total)

FIG 11 —Bilateral preparation, soleus jerks elicited by electromagnetically released myograph; right soleus, recording above, sympathectomised 23 days previously, "silent periods"—on normal side 0 07 second, on operated side 0 12 second Tension scale as in fig 8

PLATE 23

FIG 12 —Bilateral preparation, right soleus, recording above, sympathectomised 25 days previously, table fall equals 3 mm in 1 3 seconds Note the greater tension developed by the normal soleus

FIG. 13 —Same preparation as in fig 12, same amount of stretch at a faster rate, 3 mm in 0 19 second Note the greater tension developed by the sympathectomised soleus

FIG 14 —Bilateral records of clonus, sympathectomised muscle records above, rate on normal side 15 per second Fast rate on sympathectomised side approximately 50 to 60 per second. Time as in fig 11.

FIG 15A —Clinical lengthening reaction produced in sympathectomised muscle by a gradually increasing tension applied to the tendon (soleus operated 28 days previously); fall of signal indicates onset of lengthening reaction

FIG. 15B —Lengthening reaction elicited in a normal soleus by the same method These two records were made successively and are not comparable in point of time

FIG 16 —Philippson type reflex (soleus exciting—soleus reacting) Contraction of sympathectomised soleus recorded below, tension scale as in fig 12.

FIG 17 —Control myogram of soleus jerk elicited in normal bilateral preparation by electromagnetically released myograph. Taken at a relatively high initial tension and displaying the maximum observed disparity of 0 019 second in the "silent periods" of two normal soleus muscles

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Carbohydrate Metabolism in Birds. The site of the Biochemical Lesion in Avian Polyneuritis.

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(From the Department of Biochemistry, Oxford)

Recent work has defined a lowered power of oxygen uptake in certain parts of the pigeon's brain in glucose-phosphate Ringer (Gavrilescu and Peters, 1931) The partial restoration of oxygen uptake which takes place upon addition of small amounts of vitamin B_1 concentrates, excluded lowered substrates alone as the cause of the effect This could have been excluded upon other grounds, as several observers (Loebel, 1925 , Meyerhof and Lohmann, 1926) have shown that lactate will prolong the oxygen uptake of brain tissue, and it is known that the avitaminous brain has more lactate than the normal Since therefore the abnormality is concerned with the enzyme systems, it remains to be shown whether the effect is general, as would be expected from much of the previous literature upon the subject (see references in previous papers), or whether it is confined to an enzyme system for special substrates In the latter case the vitamin would be concerned with one substrate and hence one phase of metabolism , in the former it would perhaps form part of the respiratory enzyme system (Warburg, 1928 , Keilin, 1929) Holmes (1930) and Krebs (1931) have given reasons for the belief that glucose oxidation in brain proceeds via lactic acid, as the metabolism is disconnected between the glucose-lactate stages in presence of fluoride or iodoacetic acid. A lesion affecting the intermediary metabolism of carbohydrate might be confined to glucose and lactate and not found with succinate (Quastel and Wheatley, 1931) Since the matter seemed crucial, we have compared the oxygen uptakes of normal and avitaminous brains in the presence of these three substrates, succinate being regarded as typical of another system Since cyanide inhibits the uptake of oxygen for all three substrates, any difference in their behaviour in avitaminosis indicates that the vitamin acts specifically and not upon the indophenol oxidase Our results show that a tissue may behave normally to succinate and yet abnormally to lactate, and are therefore consistent with the view that vitamin B_1 deficiency is connected essentially with the intermediary metabolism of carbohydrate

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Methods

Oxygen uptakes have been measured as in previous experiments upon pigeon's brain finely minced with bone spatulas By organising the work it has proved possible to set up as many as 18 apparatus (Barcroft-Dixon type) with the necessary weighings, etc , in 40–50 minutes from death of birds, thereby reducing as far as possible the time for which the tissue stands before observation There is no indication that the difference between 35 and 55 minutes affects the results Observations were first made upon oxygen uptake in the presence of air, later the bottles have been filled with oxygen to avoid possible objections to the work upon the grounds of uneven saturation of the tissue * As will be seen from some results with air recorded in the appendix, the uptake with oxygen and lactate shows an increase of about 25 per cent as compared with air † With the improvement in technique, we have latterly been able to get as a maximum 12 samples from cerebrum, and 8 from the optic lobes/rest, thereby facilitating comparisons upon the same brain Amounts of 0 050–0·110 mg of tissue (wet weight) have been used Slightly greater accuracy would have been obtained by doubling the amount of tissue, but this would have interfered with the undoubted advantage of obtaining as many comparisons as possible upon individual brains The optic lobes/rest as before included all parts of the brain other than the cerebellum lying below the cerebrum Bottles were weighed before and after introduction of the samples. Immediately before fitting to the apparatus the tissue was broken up with a glass crusher, so as to prevent the balling of the tissue, which had sometimes spoilt the previous experiments Observations were made at 38° C , 12 minutes was allowed for equilibration before taking first readings Constants were determined at 38° C ± 0·1° C under the direct conditions of the experiments

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* No attempt was made to free the oxygen from traces of air, as controls showed that a reduction of the oxygen to 80 per cent gave the same results as 98 per cent.

† There is therefore no evidence that increased pressure of oxygen is toxic to these particular systems This needs reference in view of U v Euler's finding (1930) that some tissue catalysts function better with low concentrations of oxygen, and also of the known fact that high oxygen pressures are toxic to animals.

Chemicals

Glucose has been an A R specimen No difference was found upon one occasion upon substituting a highly purified glucose used in Streprothrix Cor work *

Succinate —Two specimens have been used, a sodium succinate (Kahlbaum) and a pure specimen of succinic acid (De Haan), m p 184 5° C In the latter case the succinic acid was added to the Ringer-phosphate solution in appropriate amount before bringing to the p_H of experiment p_H 7 4, with NaOH No difference was observed between the two samples

Lactate (Na) — This was prepared from recrystallised racemic Zn lactate by the method of Meyerhof and Lohmann (1926), and in most cases was used upon the day of preparation The concentrations were checked upon aliquot samples by the method of Friedmann, Cotonio and Shaffer as modified by Friedmann and Kendall (1929) The Ringer-phosphate was similar to that previously used

Vitamin samples were prepared with control tubes as previously described Vitamin B_1 concentrate of activity 50 γ (pigeon day dose) was mostly used, though the result was checked in one case with one of 12 γ

The polyneuritic birds were prepared as usual, and were for the most part birds which had been upon vitamin B_1 tests As a rule they had shown opisthotonus symptoms for some hours before use, which ensured that the depletion of the vitamin factor in the brain was advanced Details of the cases are not recorded as they present no new feature

Discussion upon Methods

The choice of " minced tissue " needs some comment As stated previously, tissue sections have not been found satisfactory with these brains They have the further disadvantage that the enzyme systems studied would not be sufficiently mixed to give reasonably regular results The figures given in the tables are mostly the mean of duplicate estimations In cases of unsatisfactory duplicates or single readings, the values are bracketed

Out of 50 estimations of the residual oxygen uptake, 30 gave a final difference in the reading of 10–20 scale divisions, the average difference between duplicates being 7 per cent. (0–14 per cent in the extreme) In 20 determinations with a scale reading of 20–30, the average difference was 4 per cent This is so close to the theoretical limits, namely one division, that it seems the difference was mainly due to the error of reading Results therefore with duplicates differing by more than 14 per cent. for 10–20 divisions or 8 per cent. for 20–30 have been marked with a bracket

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* We are much indebted to Dr Reader for this specimen.

In lactate, glucose and succinate determinations the difference between duplicates is usually more than would be expected from errors in reading alone In 40 succinate determinations where the scale reading was from 50 to 100 the average difference between duplicates was 5 per cent In 52 lactate determinations with a scale reading between 10 and 50 divisions the average difference was 7 per cent

All results where the duplicates differed by more than 10 per cent in the case of succinate, or 14 per cent in the case of lactate, have been marked with a bracket The glucose results given are the mean of duplicates agreeing within 10 per cent, except in the case of the one marked.

It is curious (and as yet unexplained) that the differences between duplicates with added substrate are greater than for residual oxygen uptake The differences lie outside the errors of reading, and cannot be due to uneven absorption of CO_2, as the precautions of Dixon and Elliott (1930) were always observed They lie therefore in the tissue itself. At first we thought that they were more marked with avitaminous tissue, but this view is not supported by the present data

Experimental

Approximately 100 brains have been studied The following comparisons have been performed as far as possible upon the same brains, residual oxidation (with no addition beyond the Ringer-phosphate solution), Ringer-phosphate with addition of glucose, lactate or succinate

Before giving the comparative results, we must discuss two factors which influence interpretation of the figures, namely the residual oxygen uptake (tissue without added substrate) and the question of the degree of saturation of the enzyme system

Residual Oxidation

It is well known that the residual oxidation may vary owing to the fluctuations in the amount of substrates present at the moment of death In the work of Abderhalden and Vlassipoulos (1931), only the residual oxygen uptake in air has been measured It is difficult without the knowledge of all their figures to ascertain upon what the conclusion of lowered oxygen uptake is based, —24 per cent for avitaminous as compared with normal brains In the few previous experiments, reported by us in bicarbonate Ringer, the differences were small

Table I and fig. 1 show that in presence of oxygen, the values for the residual oxygen uptake of normal tissue amounts to 865 mm ^{3}/gm /hr for cerebrum and for avitaminous, 770 mm.^{3}/gm./hr with an average difference of —11 per cent. for avitaminous The optic lobes gave an average of 650 mm ^{3} for normal and 610 for avitaminous with an average difference of 6 per cent.

Table I.—Residual Oxygen Uptake (without the addition of substrate), mm³/gm /hr

Cerebrum				Optic lobes/rest			
Normal		Avitaminous		Normal		Avitaminous	
Experiment	O_2 uptake	Experiment	O_2 uptake	Experiment	O_2 uptake	Experiment	O_2 uptake
181	795	179	(845)	187	(610)	182	770
186	(1050)	180	675	201	(760)	183	710
187	515	182	(915)	212	(625)	195	(550)
201	895	183	950	213	625	217	630
207	1070	193	(720)	215	(740)	218	685
209	1000	195	(740)	225	715	219	725
212	1020	196	820	247	625	220	(590)
213	775	217	835	257	480	222	700
215	805	218	(850)			223	730
225	(1190)	219	720			224	510
228	(760)	220	765			226	540
237	735	222	710			227	690
239	635	223	940			229	(460)
240	905	224	740			231	(495)
242	895	226	655			232	(585)
247	805	227	(730)			233	490
		229	665			236	550
		232	(850)			243	(625)
		233	(840)			245	(620)
		234	(950)			248	(520)
		235	675				
		236	730				
		238	720				
		243	760				
		245	620				
		248	725				
		249	685				
Average	865	Average	770	Average	650	Average	610
Standard deviation	170	Standard deviation	90	Standard deviation	85	Standard deviation	90

Reference to the scatter diagram shows that the distribution is so nearly the same that the average differences are not convincing and are actually not statistically significant. Hence for brain without added substrate, we cannot confirm the results of Abderhalden and Vlassipoulos, as to significant differences between normal and avitaminous tissue.

Owing to the obvious variation in the residual oxygen uptake, some discussion is required of its bearing upon the response to different substrates. As a matter of convention, we have compared the oxygen uptakes over the first hour. During this time the rate of oxygen uptake falls off rapidly with Ringer-phosphate alone, and less rapidly in the presence of substrates. Our curves have not differed in essential particulars from those of other workers Over the hour period the effects are composite, the components being the residual uptake of oxygen by the tissue, and the oxygen uptake due to the substrate used

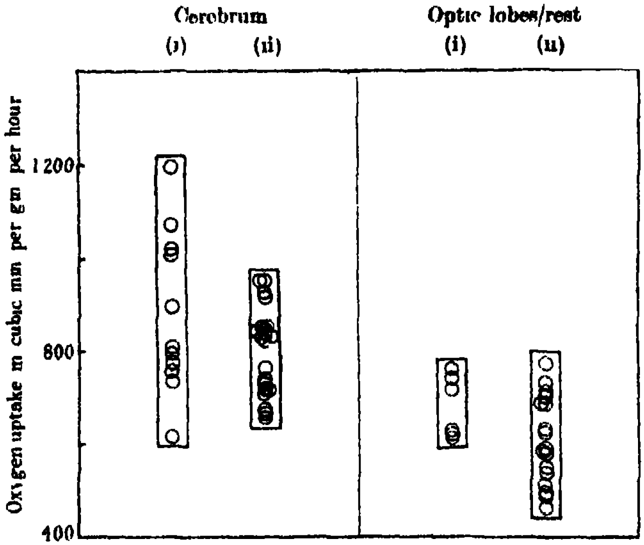

Fig 1 —Oxygen uptake without added substrates (residual oxidation) (i) Normal, (ii) Avitaminous

The observed residual oxygen uptake (L + R) equals the residual oxygen uptake for substrates other than lactate (R) (under our conditions of constant feeding, etc , probably not very variable, and that due to preformed lactate (L)) This contributes the largest share to the residual uptake

That this is so can be shown in various ways The affinity constant for lactate is of the order 0·007 M (Haldane, 1930) This holds under our conditions, since extrapolation of two of our curves, one avitaminous and one normal both gave the value 0·008 M. Hence quite small amounts of lactate are sufficient to influence the oxygen uptake Further, as Krebs (1931) and Meyerhof and Boyland (1931) have shown poisoning with mono iodoacetate much reduces the oxygen uptake as well as the lactate present In two experiments with brains poisoned in this way we found a reduction in oxygen uptake of 30 per cent and 50 per cent

Hence it is safe to assume that the observed residual oxygen uptake (L + R) is due to a small amount (R) of unknown substances and a larger amount of lactate preformed (L). A sufficient number of observations should give the limits for residual uptake under our conditions. In the curves given below, the actual figures are plotted without subtraction.

There is another way of regarding the results, which gives substantially the same answer. Since glucose is rapidly glycolysed to lactate, any preformed glucose in the tissue can be neglected If R is regarded as constant, the uptake in presence of an amount of lactate sufficient to saturate the system forms a fair measure of the oxygen uptake for lactate Any preformed lactate in the tissue merely adds to that which is present On the other hand, since large amounts of succinate must be present to saturate the succinate system, and since there is no evidence that much preformed succinate is present, the above procedure will not be accurate for succinate In this case, we should theoretically subtract the observed residual uptake from the uptake in presence of succinate in order to approximate to the uptake for succinate alone In this case, of course, the assumption has to be made that the metabolism of the tissue is not substantially different under the two conditions

Guha (1931) has estimated the amount of lactic oxidase present in rat's brain by comparing the oxygen uptake with and without added lactate This is not an absolute method unless the preformed lactate can be shown to be constant Such a method really measures the difference between two concentrations of lactate, one of which is unknown

Saturation of Enzyme System

In the previous experiments, no mention was made as to whether the enzyme system was saturated at the concentration of glucose employed, it was our intention to work under conditions approximating to the normal to which such tissues would be exposed Actually experiment showed that increase in glucose up to 1 per cent and even 3 per cent produced practically no increase For purposes of comparison, estimations of the O_2 uptake must be made either at maximal saturation or if not at some defined degree of unsaturation Fig 2 shows that the variation with different lactate concentration becomes negligible over 10 mg lactate 3 c.c (0 037 M) In this region, changes in lactate concentration of 0·5 mg. produce variations of not more than 50 mm ^{3}/gm per hour. Our comparisons were made at 0·037 ± 0·002 M lactate concentration With succinate the position is different. As the succinate concentration is

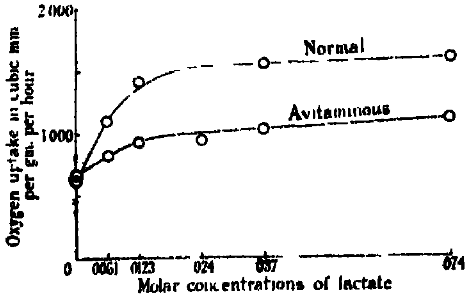

FIG 2 —Oxygen uptake for normal and avitaminous brain with varying concentration of lactate

increased from 0–100 mg increase in oxygen uptake takes place up to a maximum of 4500 mm ^{3} for cerebrum, 3000 mm ^{3} for the optic lobes At the highest concentration the solutions are, of course, enormously hypertonic, but this does not seem to affect the smoothness of the curves relating rate to concentration. Fig. 3 obtained in an experiment upon the cerebrum of one bird shows the variations to be expected upon increasing the concentration from 9-90 mg succinate per 3 c c (0 0185–0 185 M)

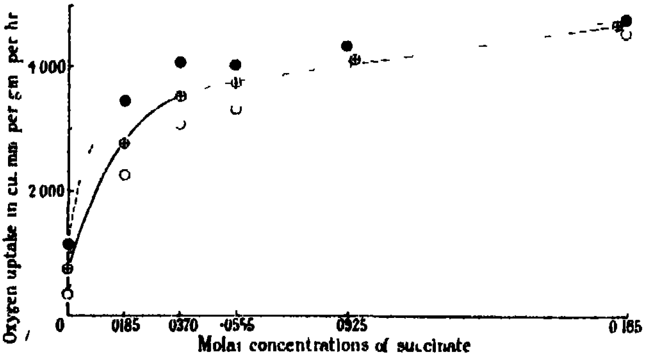

FIG 3 —Oxygen uptake with varying concentration of succinate. ● - - velocity over first 10 minutes ⊕ —— velocity over whole time ○ . velocity over last 10 minutes

The upper curve shows the oxygen uptake per gram of tissue per hour calculated for the period 0–10 minute The middle curve shows the corresponding uptake for 0–60 minutes and the bottom curve for 50–60 minutes The falling off of the oxygen uptake is caused by three factors . (1) The gradual decrease in the residual oxidation , (2) the gradual decrease in succinic acid concentration (this is negligible at high concentrations) , (3) possible destruction or blocking of the enzyme systems All these factors will be at a minimum for the first period Reading from the figure, Table II is obtained

A Michaelis constant of approximately 0 0096 M is given for the succinoxidase under these conditions Widmark (1921) using muscle extracts obtained a Km 0 001 M for succinoxidase, *i.e.*, ten times less We have made most of our comparisons between 0 0185 M and 0 0555 M The region of maximum uptake has been avoided owing to the enormous hypertonicity there

Normal and Avitaminous Tissue and Behaviour to different Substrates

Kinnersley and Peters (1930) found that in birds with threatening symptoms, the changes in lactic acid tended to be most marked in the optic lobes/rest.

Table II

Period 0–10 minutes Residual oxidation 1160 mm^3/gm./hr Equation used $K_m = x\,(V/v - 1)$ (Haldane, 1930)

Succinic acid conc (M) (x)	O_2 uptake, mm^3/gm /hr	O_2 uptake — residual oxidation (v)	K_m
0 0093	2900	1740	0 0094
0 0185	3450	2290	0 0102
0 0279	3800	2640	0 0095
0 0370	4045	2885	0 0085
0 0463	4160	3000	0 0083
0 0555	4220	3060	0 0089
0 0925	4335	3175	0 0111
0 183	4715	3555 (V)	—

As the symptoms persisted, changes also were found in the cerebrum Most of our cases therefore could be expected to show lactic acid changes in the cerebrum, as well as the rest The degree of lowering of the oxygen uptake with glucose as substrate (as compared with the figures in our previous paper) can be used as some measure of the extent to which the condition has advanced

Fig. 4 summarises all the results for lactate and figs 5 and 6 for succinate Table III shows the data for four typical experiments with comparison of glucose and lactate upon the same tissue, and three for glucose and succinate,

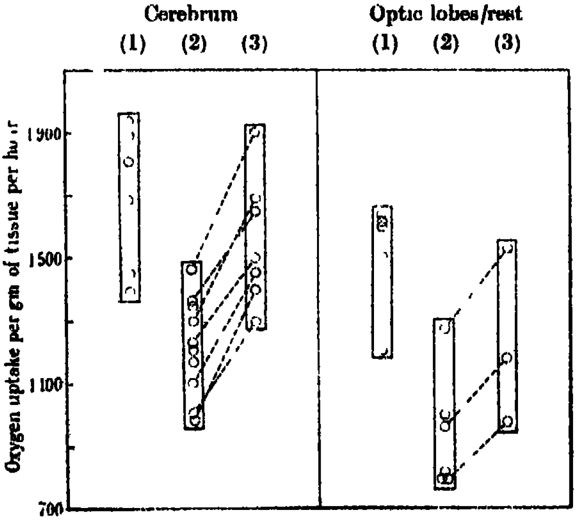

Fig 4.—Oxygen uptake in presence of 0 037 M lactate, for (1) normal, (2) avitaminous and (3) avitaminous brain with addition of vitamin B_1 concentrates.

and averages for normal and avitaminous brains with succinate as substrate. A series of 25 experiments of this nature have convinced us that in cases where there is a marked lowering of the reaction of the tissue to either glucose

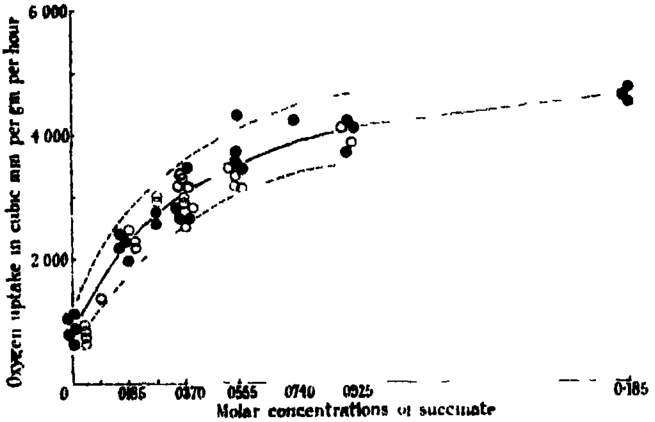

FIG 5 —Oxygen uptake for normal and avitaminous cerebrum in presence of varying concentration of succinate • normal brains ○ avitaminous brains

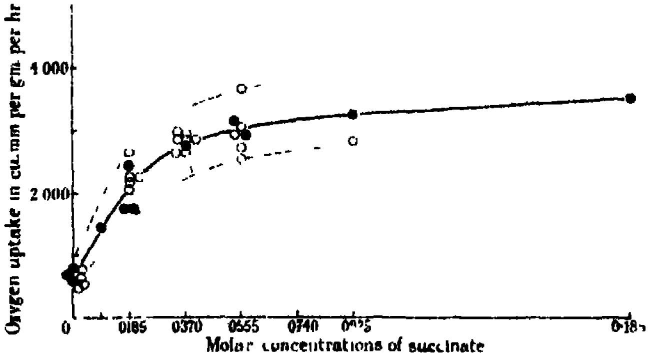

FIG 6 —Oxygen uptake for normal and avitaminous optic lobes/rest in presence of varying concentration of succinate • normal brains ○ avitaminous brains

or to lactate, the uptake of oxygen with succinate is practically normal The tissue is normal to succinate and abnormal to glucose and lactate

It will be interesting to explore these differences further with other substrates, but we think that they prove the contention that in two oxidase systems, both of which are inhibited by cyanide, the lesion in avitaminosis is in the glucose-lactate system, which indicates that it is concerned with the oxidative metabolism of lactate, how directly cannot yet be stated. Previous work upon oxidase systems in avitaminosis, to which reference has been made

Table III—Oxygen uptake, mm.^{3}/gm /hr

A Glucose-lactate

Type	Experiment	Cerebrum.			Optic lobes/rest		
		Ringer only	Glucose 0 25 per cent	Lactate 0 037 M	Ringer only	Glucose 0 25 per cent	Lactate 0 037 M
Normal	213	775	1645	1795	630	(1410)	1600
Normal	215	805	1955	2275	(755)	1460	(1620)
Avitaminous	217	830	1330	1220	630	945	790
Avitaminous	218	(850)	1355	1350	685	870	945

B Glucose-succinate

Type	Experiment	Cerebrum			Optic lobes/rest		
		Ringer only	Glucose 0 25 per cent	Succinate	Ringer only	Glucose 0 25 per cent	Succinate
Normal	225	1195	1955	(0 0185 M) 2390 (0 0555 M) 3500	715	-	(0 0370 M) 2965
Avitaminous	183	945	990	(0 0370 M) 3325	715	1200	(0 0185 M) 2180
Avitaminous	229	665	1095	(0 0370 M) 2800	460	—	(0 0370 M) 2990

C Succinate Averages

Concentration	Cerebrum		Optic lobes/rest	
	Normal	Avitaminous	Normal	Avitaminous
M				
0·0185	2160	2310	2220	2270
0 0370	2940	3020	2760	2770
0 0555	3770	3340	3050	2990

in other communications, has been mainly upon other tissues Results with these are likely to be complicated by the fact that tissues other than the brain[*] are apt to make lactate after mincing, and by the difficulty of deciding how far the avitaminous lesions have advanced In the case of brain the nervous symptoms form an indication of this But it seems to us very probable that some of the controversy in previous work has been due to the choice of either no substrate or of dissimilar substrates or of methylene blue methods; Drummond and Marrian (1926), for instance, used succinate, which we have shown in brain behaved normally, and hence would account for their view that the avitaminous tissues were not changed It seemed that the matter could be settled if it was shown that the vitamin B_1 concentrates had a greater effect upon the lactate than upon the glucose oxidations when added as previously

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[*] With brain, formation of lactate from tissue has been found to stop within a few minutes of death.

Addition of Vitamin B_1 Concentrates *

The results of experiments are summarised in Tables IV and V In eight experiments with cerebrum, there is an increase of 20–32 per cent in the amount of oxygen taken up in the hour period, the effects are therefore much greater than with glucose (+ 12 per cent) The results for the residual uptake, Table V, are practically as large as those in the presence of glucose, which must be due to the traces of preformed lactate present In striking contrast is the effect with succinate Here the slight increase is generally equivalent to the effect of the vitamin upon the residual oxidation (presumably the preformed lactate), except in the case of experiment 226, which we are unable to explain, but it is clearly an exception to the general rule We would emphasise that the effect

Table IV —Avitaminous Brains (Lactate) 0 037 M O_2 uptake mm ^{3}/gm /hr

Experiment	Cerebrum		Optic lobes/rest	
	Without vitamin	With vitamin	Without vitamin	With vitamin
193	(1450)	—	990	—
195	1170	—	800	—
214	1460	(1900)+30 per cent	(1270)	1530+20 per cent
216	1000	1300 +30 per cent	—	—
217	1200	—	790	970+23 per cent
218	1350	(1650) +22 per cent	950	1180+24 per cent
219	(1100)	(1450)+32 per cent	—	—
232	1300	1690 +30 per cent	810	—
233	980	1400 +42 per cent	—	—
234	1210	1500 +24 per cent	—	—
Averages	1220	1560 + 28 per cent	935	1230+22 per cent
Standard deviation	160	—	168	—

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* As previously, we are taking the simple view that the "lactate" factor in the concentrates is vitamin B_1 This cannot be settled until crystalline vitamin is available.

of the addition must be catalytic In experiment 217, not more than 5 γ solid weight added to the 3·0 c c caused an extra increase of oxygen of approximately 120 γ The results for lactate perhaps explain some of the curious anomalies of the cerebrum in the experiments with glucose and vitamin concentrates They would depend in part upon the rate of lactate formation from the glucose, i e , upon the integrity of the glycolytic system

Table V —Avitaminous Brains O_2 uptake mm ^{3}/gm /hr

Cerebrum

Experiment	Ringer				Succinate (0 037 M)			
	Cont	Vit	+	Per cent.	Cont	Vit	+	Per cent
226	660	820	160	24	3170	3330	160	5
227	(730)	910	180	25	(2980)	3120	140	5
229	665	(760)	95	15	2800	2800	0	0
230	790	935	145	19	3315	3335	20	0
231	-	—	—	—	—	—	—	—
219	-	-		—	—	—	—	—
Average increase			145	21	—		80	2 25

Optic lobes/rest

Experiment	Ringer				Succinate (0 037 M)			
	Cont	Vit	+	Per cent	Cont	Vit	+	Per cent
226	500	540	40	9	2375	2780	405	17
227	640	690	50	8	2910	3055	145	5
229	460	600	140	30	2990	2840	(−150)	(−5)
230	(565)	655	90	16	2855	2905	150	5
231	(495)	600	105	20	(2290)	2515	225	10
219	725	—	—	—	2970	3080	110	4
Average increase			85	17			130	5 5

Cont = control

Effect of Vitamin B_1 Concentrates upon Normal Brain

Table VI shows the action of the same vitamin concentrates upon seven normal birds It will be seen that effects obtained, except in one case, are slight and of a different order to those in Table IV, averaging + 4 per cent. Hence the normal tissue is practically saturated so far as the factor supplied

Table VI —Normal Brains

Vitamin B_1 concentrates (1/10 pigeon dose) Substrate, lactate (0·035 M).

Experiment	Cerebrum			Optic lobes/rest		
	Cont	Concentrate	Per cent	Cont	Concentrate	Per cent
208	1390	—	—	1200	—	—
213	1810	—	—	1660	—	—
237	1660	1630	− 1	—	—	—
240	1810	1820	Nil	1270	1520	+ 20
242	1590	1750	+ 9	1590	1490	− 6
247	1640	1850	+ 12	(1170)	1270	+ 9
255	1550	1660	+ 7	(1450)	1390	− 4
256	1690	1720	+ 1	1320	1390	+ 5
257	1480	1520	+ 3	1200	1210	+ 1
Average	1620	1710	+ 4	1380	1380	+ 4
Standard deviation	132	—	—	178	—	—

by the concentrates is concerned The vitamin therefore is much more active with the avitaminous than normal brain in presence of lactate The effect of vitamin extracts on avitaminous brain appears to be completely at variance with the final conclusion reached by Galvao and Cardoso (1930), namely, that the lessening of oxidation in avitaminosis B_1 is due exclusively to a state of partial inanition This general conclusion is reached as a result of a series of experiments upon the oxygen uptake of whole pigeons, and of the reduction of methylene blue by muscle and liver However interesting these facts, they only show that certain systems behave the same in an avitaminous and starved animal The real point at issue is why the avitaminous animal voluntarily starves itself This we believe is more likely to be decided by experiments upon the brain where some indication of physiological abnormality exists

Discussion

A prominent aim in these investigations has been the hope of correlating pathological symptoms with biochemical changes, and so helping to co-ordinate biochemical enzyme studies with the behaviour of the brain in life It may be argued that our conditions are too artificial to admit of application to the tissues in the living brain , this argument is certainly valid in the sense that we do not know what modifications are imposed on the enzyme systems during life But it is difficult to believe that the difference found in these cell suspensions do not indicate changes of some similar type in the living tissue

In this paper the proof is given that in the pathological condition of polyneuritis in birds, a prominent lesion in the systems studied is not one which affects all oxidase systems, but that it is associated with lactate, the oxygen uptake *in vitro* being increased in the presence of this substrate upon addition of vitamin concentrates Hence, the vitamin is not part of the respiratory enzyme system (Warburg) or of the cytochrome (Keilin), supposing that they are not identical It is therefore incorrect to consider it as a general "Atmungstoff" (*cf.* Abderhalden and Vlassipoulos, 1931), though our experiments support the theory that the vitamin is an integral part of one oxidase system It may therefore be in that sense acting as an "Atmungstoff" We would point out, however, that this is by no means proved There are still alternative explanations for the undoubted increase in oxygen uptake which takes place when vitamin is added to avitaminous brain in the presence of lactate These are being now investigated That the factor in the vitamin concentrates is not merely acting like methylene blue in catalysing the uptake of oxygen with lactic dehydrase appears to follow from the experiments of Stephenson (1927) on the soluble lactic dehydrogenase from *B coli* She could not restore oxygen uptake by adding yeast extracts It is clear that the strongest support is given to the inferences drawn by Kinnersley and Peters (1930) from experiments which showed localised accumulation of lactic acid in the pigeon's brain in these conditions, namely, that these were due to the lack of some factor in the tissue (B_1) connected with the oxidative removal of lactic acid and is consistent with Fisher's experiments (1931) It seems that the experiments are also the best evidence yet obtained for the theory that vitamin B_1 is a factor in the intermediary metabolism of carbohydrate The accompanying diagram is put forward as a working hypothesis of the present position reached (fig. 7)

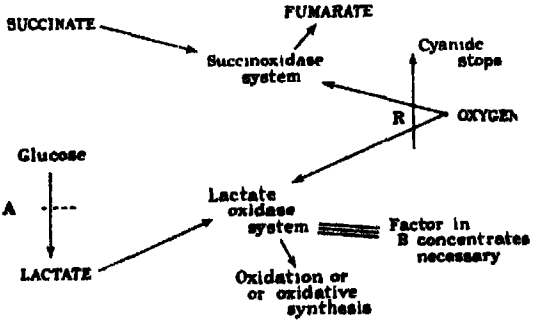

FIG 7.—Diagram to illustrate present position reached as to site of action of factor in B_1 concentrates. A, blocked by fluoride (Loebel, Holmes) or by iodoacetate (Lundsgaard, Krebs). R, respiratory enzyme system

Conclusions

1 The oxygen uptake of minced polyneuritic pigeon's brain without added substrate (residual oxidation) is little different from that of normal

2 In presence of added lactate, there is an average diminution for cerebrum of — 33 per cent and optic lobes of — 45 per cent, whereas in presence of succinate the behaviour of normal and abnormal tissue is the same

3 The specific "lactate" abnormality is largely restored by addition of vitamin B_1 concentrates, which produce negligible effects upon the normal.

4 Hence it is inferred that the biochemical lesion is not general in B_1 avitaminosis, but is specific for the glucose-lactate enzyme systems, and therefore expresses change in the intermediary metabolism of carbohydrate

Our thanks are due to Mr H W Kinnersley for specimens of the concentrates used in this research, to the Government Grants Committee of the Royal Society, and to the Medical Research Council for grants towards the cost of the work We are also grateful to Dr Walker for his help with the apparatus used

APPENDIX

The following table gives experiments with lactate in air, and shows for cerebrum that the average values are some 25 per cent lower than those in oxygen It will be noted that the average value for optic lobes and the rest of the brain considered separately is 1340 and 1210 mm^3, which indicates a lower capacity on the part of the "rest" than of the "optic lobes" In the experiments

Table VII —Experiments in Air Normal Brains
Na Lactate (approx 10 mg /3 c c)

Experiment	Cerebrum	Cerebellum	Optic lobes	Rest
25	1550	850	1450	1320
26	1600	1470	—	1170
29	1480	1570	1190	1200
32	1490	1260	1270	1010
33	1350	1280	1360	1310
35	1435	1820	1422	1290
36	1435	—	—	—
37	1350	—	—	—
Average	1460	1370	1340	1210
Value for glucose in air* — Average	1260	1032	1185	997

* From previous work

given elsewhere in the paper, any differences of this kind were sacrificed by mixing these two tissues in order to obtain sufficient tissue for average comparisons It will be seen that the general level of oxygen uptake is greater in presence of lactate than with glucose This conclusion can also be drawn from a comparison of the experiments with glucose and lactate in oxygen

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The Influence of Coloured Surrounds and Coloured Backgrounds on Visual Thresholds.

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(Communicated by Sir Charles Sherrington, F R S —(Received December 31, 1931)

(From the Department of Physiology, London Hospital Medical College)

The present observations were a development of previous work on the effect of lights of different wave-lengths on each other when superimposed on the same retinal area (Roaf, 1928) The methods used were (*a*) to measure the threshold of a test light when shining alongside a modifying light, and (*b*) to measure the threshold when the modifying light shone on the same part of the retina as the test light, thus forming a background for it It was assumed in that paper that by measuring the threshold of the test light when shining alongside the modifying light the observer's eye would be in the same state of dark adaptation as when the two lights were superimposed, but it was pointed out that adaptation might be local and that "scattering" of light might have affected the results (Roaf, 1928, p 254)

Various arrangements of test and modifying light have now been studied to determine to what extent they will alter the threshold of the test light A preliminary account of these experiments has been published (Roaf, 1931)

The threshold of a test light may be changed by a modifying light in one of four ways, namely —

A Change in area of the aperture of the pupil

B Change in sensitivity of one region of the retina as the result of light falling on another region

C Interaction via the nervous system when the test light is presented to one eye and the modifying light to the other

D Local effects due to superposition of one light on another

A Change in the area of the aperture of the pupil can be excluded from influencing the experiment by the use of an "artificial pupil" with a narrower aperture than that of the pupil of the eye employed in making the observations The insertion of an artificial pupil for vision outside the pupil requires elaborate apparatus which was not considered necessary for the present observations The effects of changes in aperture of the pupil have been tested by foveal readings with and without an artificial pupil It was found that the illuminations used had small influence on the area of the pupil (see Tables I (*a*) and IV (*a*))

B (α) The influence of one part of the retina on another may be purely optical On looking at a small source of light the image is not sharply demarcated from a region of non-illumination, but there is always a surrounding area of decreasing illumination Further, there is always some irregular diffraction at the optical surfaces and scattering of light in the eye media which tend to throw a certain amount of " stray " light on parts of the retina outside the image, and even remote from the image This " stray " light is particularly noticeable when the eye is dark adapted On looking at a small source of light the whole eye then seems to be illuminated by a pale bluish haze The effects of spread of illumination cannot be wholly avoided, except by using the test light for one eye and the modifying light for the other

B (β) When light falls on a part of the retina the nerve impulses, thus set up, may interact with those from another part if there are synaptic connections between the cells of the retina This kind of spread has been ably discussed by Adrian and Matthews (1928) for the eel's eye, and studied by Granit and his co-workers (1930, 1931) in the human eye Allen (1923 1926) has described an interaction between the two eyes which he ascribes to a " sensory reflex," but the effect of a stimulus to one eye on the other eye may be due to interaction in the central nervous system

C Central interaction can be studied by binocular vision. Parsons (1924, p. 54) states that " dark adaptation in one eye has no effect upon the other " Sherrington (1904) found a slight difference in the rate of revolution at which flicker disappeared when the two eyes were illuminated simultaneously from the rate required when they were illuminated alternately

D Local effects due to superposition were the object of a previous study (Roaf, 1928) They are probably the result of photochemical action

PART I

As the processes described under headings A, B and C were studied by the same apparatus, the experimental methods and results will be described in one section

Experimental Methods.

The measurements are done in two stages. In the first place, the absolute threshold was measured when no other light is being presented to the eye.

In the second place, the differential threshold is measured when the modifying light is being simultaneously presented with the test light

The small light of which the threshold is being measured will be described as the test light, and the larger field of illumination which affects the test light will be described as the modifying light The test light subtended a visual angle of 1° 50′ and the modifying light subtended an angle of 43°

The source of light was split up into its component wave-lengths by means of a spectroscope used in previous researches (Roaf, 1924) The pure spectrum was caused to fall on the diaphragm (A) with a disc aperture An image of the disc aperture was focussed on the ground glass screen (D) by means of the lenses (B_1, B_2) and a totally reflecting prism (C)

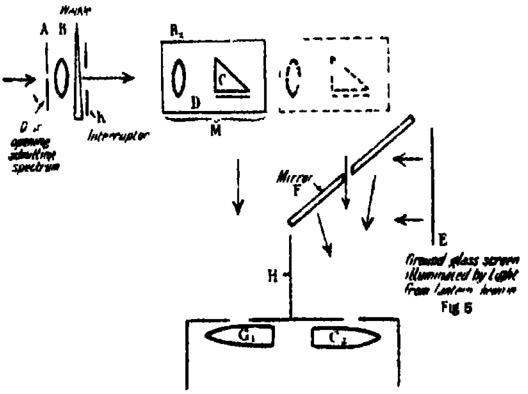
Wedge
A
B
B_2
C
D
h
Disc opening admitting spectrum
Interrupter
M
Mirror F
E
Ground glass screen illuminated by light from lantern
Fig 5
H
G_1
C_2

FIG 1 -For description see text. The modifying light is furnished by the apparatus in fig 5 without the totally reflecting prism and the optical parts related to it in that figure

The lens (B_2), the prism and the ground glass were together mounted on a small movable base (M)

The modifying lights were furnished by colour filters used in a previous research The "red"* screen transmitted a band from the extreme "red" end of the spectrum to about 6600 Å. and at full intensity was about 2500 times the threshold intensity The "green" screen transmitted a band from 4960 to 5560 Å. and at full intensity was about 2500 times the threshold intensity The "blue" screen transmitted a band from 4170 to 4790 Å and at full intensity was about 1000 times the threshold intensity

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* The terms "red," "green," etc, will be used for brevity denoting light of such wave-length as would give rise to the sensation of red, green, etc, respectively, to a normal person.

The means by which the modifying light was projected on the ground glass screen (E) is shown in fig 5, but the totally reflecting prism and optical parts related to it in that figure were not present A gas-filled opal electric light was contained in a ventilated light-proof box in one side of which the colour filter could be fitted The light transmitted through the colour filter passed through a shutter by which the intensity of illumination could be altered, then through a lens by which an image of the opal bulb could be formed on the ground glass screen (E)

The light from the ground glass screen, E, was reflected by a mirror (F) set at 45° to E In this mirror was a hole drilled at an angle of 45° so that the hole was centred on the optical axis of the light passing to the observer's right eye Between the stereoscopic lenses (G_1, G_2) was a screen (H) to prevent cross illumination A chin rest was provided inside the box surrounding the stereoscopic lenses Precautions were taken to limit extraneous illumination from other parts of the dark room The test light was interrupted by a revolving sector shown at K because it was found to increase slightly the accuracy of the observations

By shifting the unit, M (consisting of lens B_2, prism and ground glass) to the right it was possible to view the test light through the hole in the mirror (position shown by interrupted lines in the diagram) so that both lights shone in one eye, the test light being visible in a dark area at the centre of the modifying light, the left eye being open but entirely unilluminated

By shifting the unit, M, to the left (position shown by continuous lines in the diagram) the test light would shine in the left eye and the modifying light in the right, in which case the images would combine in a single visual field owing to action of the stereoscopic lenses, G_1 and G_2

When observations were being made by regions outside the fovea, a fixation light was used The fixation light was produced by a neon lamp enclosed in

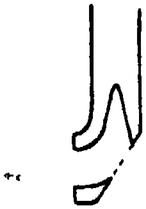


FIG 2—Section through end of fixation light showing how the tube is fused on to the rod. The interrupted line shows the angle at which the back of the tube is ground off so that light can pass through from behind. The lower margin of the tube is shown at the lower end of the interrupted line

a light-proof case, through which passed a glass rod 3 mm in diameter The light passed along the rod by internal reflection and the rod was wrapped in black tape to prevent the escape of stray light The end of the rod was fused to a glass tube which was bent at a sharp angle The convex part of the bend was ground off to give a surface at 45° to the rod and the surface polished The cut end of the tube was ground and polished in a plane parallel to the direction of the rod On viewing the tube perpendicular to its cut, polished surface, the observer sees a feeble orange light in the form of a ring (due to internal reflection from the rod to the walls of the tube and from the 45° surface towards the observer) Another light of small area can be made to appear in the centre of the ring because the convex part of the tube had been ground off

Fig 3 shows the fields for the right and left eye when both lights, the test and modifying lights, shine in the same eye (upper diagram) and when the lights shine in opposite eyes (lower diagram) The position of the fixation light when arranged in each case for extra foveal vision is shown at the right of the modifying light

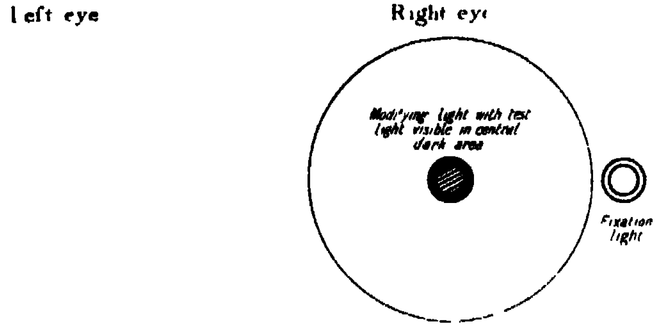
Most of the readings were made by one observer Other observers made readings, however, and of these latter two had a long period of training

When measuring the threshold of a light it is necessary to keep the direction of the eye fixed For those observers in whom the fovea is the most sensitive part of the retina for "red" the absolute threshold for "red" is easy to measure With "green" and "blue"* on the fovea the case is different and special precautions have to be taken When these lights are reduced down to the threshold at the fovea the visual axis tends to drift from the fovea The light then reappears and, consequently, the threshold measured is lower than if the visual fixation were preserved. With practice the visual axis can be moved voluntarily, making the light disappear when shining on the fovea and reappear on the other side Then the light can be varied until it will just disappear or just not disappear at the fovea

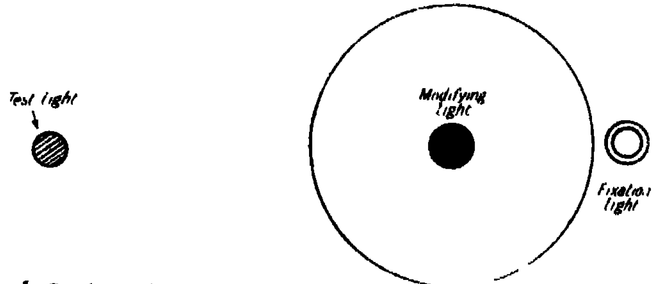
The degree of visual steadiness required is gauged by an observer's ability to look with one eye at a small spot of light, well above the threshold value, and then voluntarily to alter the direction of the visual axis until the spot disappears and remains invisible on the blind spot This incidentally illustrates the diffuseness of such illumination for there may yet still be visible a distinct halo round the blind spot

* The term "blue" is used although it may be that the actual receptor may give a sensation of violet when stimulated Violet appears, however, as if it were a mixture of red and blue

The intensities of the threshold were measured by millimetre readings of a photometric wedge. Each difference of 5 mm in the readings along the wedge corresponded approximately with a halving or doubling of the intensity of the light depending on the direction of movement of the wedge.


Left eye
Right eye
Modifying light with test light visible in central dark area
Fixation light

(*a*) For uniocular experiments



(*b*) For binocular experiments

FIG. 3. – Fields of vision arranged for readings outside the fovea. (*a*) The test light is seen by the right eye in the centre of the unilluminated area which is itself contained within the field of the modifying light. The left eye is open but unilluminated. (*b*) The test light is seen by the left eye. The unilluminated area is contained within the modifying light which is visible to the right eye. For foveal vision, if the fixation light is used it will occupy the margin of unilluminated area between the test light and modifying light in (*a*).

Sufficient time was allowed for good dark adaptation before the absolute thresholds were measured. By keeping the observing eye closed between readings it is possible to use the other eye for making adjustments and recording results.

In most cases the figures recorded were the average of five separate readings.

Table I —Increase of threshold of test light produced by surrounding illumination on foveal and extra foveal vision for the right eye of H E R

(*a*) Foveal

Test light	21 10 30 *	5 11 30 *	21 11 30	18 3 31	Average	Approximate multiple of threshold
	mm	mm	mm	mm	mm	
" Red " screen						
" Red "	2 5	6 0	9 8	11 2	7 4	3 0
" Green "	2 8	4 8	7 4	8 8	6 4	2 5
" Blue "	3 3	7 4	7 2	4 6	5 6	2 3
" Green " screen						
" Red "	2 5	5 4	7 8	11 0	6 7	2 7
" Green "	4 0	7 4	9 6	7 4	7 1	2 8
" Blue "	5 1	10 4	8 8	5 2	7 4	3 0
" Blue " screen						
" Red "	−0 1	−0 6	2 4	5 0	1 8	1 3
" Green '	1 4	1 0	4 4	2 4	2 3	1 4
" Blue "	0 7	1 6	2 8	0 8	1 5	1 2
Wave lengths of test lights						
" Red "	6527	6604	6429	6678	—	—
	6328	6478	6202	6503	—	—
" Green "	5551	5376	5422	5397	—	—
	5449	5324	5299	5275	—	—
" Blue "	4921	4819	4771	4771	—	—
	4795	4721	4669	4693	—	—

(*b*) Extra Foveal (21°)

	11 11 30	18.11 30	21 11 30	18 3 31		
" Red " screen.						
" Red "	24 2	19 6	14 0	9 4	16 8	11 9
" Green "	32 0	30 6	31 0	21 2	28 7	69 0
" Blue "	33 2	26 8	36 4	31 0	31 8	108 0
" Green " screen						
" Red "	23 2	22 2	18 0	11 4	18 7	15 9
" Green "	48 2	51 2	42 6	33 0	43 8	640 0
" Blue "	52 8	50 8	52 4	46 8	50 7	1720 0
" Blue " screen						
" Red "	12 0	18 4	9 0	5 4	11 2	5 0
" Green "	37 4	40 4	35 0	26 4	34 8	169 8
" Blue "	44 4	38 8	42 0	35 2	40 1	368 4
Wave lengths of test lights						
" Red "	6503	6678	6429	6678	—	—
	6279	6402	6202	6503	—	—
" Green '	5222	5149	5422	5397	—	—
	5173	5071	5299	5275	—	—
" Blue "	4721	4721	4771	4771	—	—
	4023	4645	4669	4693	—	—

* Artificial pupil used

Table II—Increase in threshold of test light produced by surrounding illumination on foveal and extra foveal vision for right eye of H F

(*a*) Foveal

Test light	4 3 31	19 5 31	2 6 31	Average	Approximate multiple of threshold
	mm	mm	mm	mm	
" Red " screen					
" Red "	5 0	6 4	3 8	5 1	2 1
" Green "	5 4	11 1	0 0	5 5	2 3
" Blue "	3 1	8 6	1 6	4 4	1 9
" Green " screen.					
" Red "	5 4	8 2	4 6	6 7	2 7
" Green "	7 2	11 9	1 8	6 8	2 7
" Blue "	7 3	8 8	8 4	8 2	3 3
' Blue " screen					
" Red "	3 4	1 2	0 8	1 3	1 1
" Green "	3 0	2 0	—0 4	1 8	1 2
" Blue "	4 3	2 0	4 0	3 4	1 6

(*b*) Extra Foveal (21°)

Test light	4 3 31	19 5 31	2 6 31	Average	Approximate multiple of threshold
" Red " screen.					
" Red "	5 7	2 4	3 6	3 9	1 8
" Green '	27 6	42 8	23 4	31 3	100 0
" Blue "	26 0	25 8	12 8	22 5	27 0
" Green " screen					
" Red "	9 3	8 6	8 2	8 9	3 6
" Green "	31 0	43 2	31 8	35 3	180 0
" Blue "	45 4	41 6	41 2	42 7	540 0
" Blue " screen					
" Red "	2 6	2 8	2 0	2 5	1 4
" Green "	20 6	30 8	28 2	26 5	50 0
" Blue "	37 2	31 6	27 0	31 9	112 0
Wave lengths of test lights					
" Red "	6429 6171	6853 6678	6655 6429	— —	— —
" Green "	5474 5299	5495 5352	5575 5449	— —	— —
" Blue "	4693 4567	4872 4771	4819 4721	— —	— —

Results

A Area of the pupil Judging by the experiments recorded in Tables I (*a*) and IV (*a*), the modifying lights caused a narrowing of the pupil equivalent to a decrease in illumination of from 50 to 80 per cent In other words, the effect of the modifying lights is overestimated because of the narrowing of the pupil, but this difference would amount to less than a doubling of the intensity of the absolute threshold Therefore any increase in threshold amounting to more than double of the absolute threshold cannot be accounted for by changes in the aperture of the pupil

B When we examine the influence of one region of the retina on another we must describe separately observations made with the fovea and with regions of the retina outside it

Foveal Vision with One Eye

In the fovea the influence of surrounding illumination is comparatively slight (Tables I (*a*) and II (*a*)) The threshold for the test light is raised by the modifying light to about twice or thrice that of the absolute threshold and part of this difference is due to narrowing of the pupil In the earlier experiments (not recorded in the tables) the area of surrounding illumination was used with visual angles of 14°, 29° and 43°, but the magnitude of the areas of illumination had no appreciably different effect on the threshold of the test light The aperture in the mirror subtended a visual angle of 3° 4′, thus there was a narrow darker margin separating the test and modifying lights Comparison of the magnitude of the effect of surrounding illumination with that given by actual superposition of the test and modifying lights suggests that it is possible to explain the whole of the increase in threshold, due to concentric illumination, as the result of narrowing of the pupil and of "stray" light On the other hand, it will be shown later that a corresponding rise in the threshold is caused by illumination of the opposite eye (Tables IV (*a*) and V (*a*)) In view of the narrowing of the pupil, the effect of stray light and the action of illuminating the opposite eye, the above experiments recorded in Tables I (*a*) and II (*a*) cannot be considered as furnishing evidence of interference of one area of the retina with another by interconnections between nerve cells

Vision with One Eye Outside the Fovea

The results under this heading are in striking contrast to those obtained with foveal vision There is a marked effect of a surrounding area of illumination on the visibility of the test light (Tables I (*b*) and II (*b*)) The magnitude of the increase in threshold precludes the possibility of any narrowing of the pupil being the sole cause of the increase in the threshold

It would be expected, in view of the variations in sensitivity of different parts of the retina (Baird, 1905, and Wentworth, 1930) that the effects of different coloured surrounds on different regions of the retina would be very complicated, therefore no exhaustive examination of the regional differences was undertaken It seemed, however, desirable to make some measurements at different degrees of eccentricity The combination, which had been found to give the greatest effect at 20° ("blue" test light with "green" modifying light) was used, measurements were made in a horizontal plane on the temporal area of the retina The results are given in Table III and in fig 4.

Table III —Increase of threshold of test light produced by surrounding illumination with different eccentricities of vision for right eye of H E R

Fixation	9 10 31	12 10 31	13 10 31	20 10 31	20 10 31	Average	Approximate multiple of threshold
	mm	*mm*	*mm*	*mm*	*mm*	*mm*	
Foveal	5 8	8 4	6 5	9 6	10 4	8 1	3 3
6° 9′	25 4	36 2	32 2	30 8	36 2	32 2	115
12° 19′	36 4	38 6	41 8	45 0	40 8	40 5	475
18° 27′	47 2	48 0	47 8	49 0	47 6	47 9	1150

C *Binocular Vision*

By means of the stereoscopic lenses it is possible to combine the image of the test light in the left eye with that of the modifying light in the right eye By suitable adjustment of the unit M, fig 1, consisting of lens, prism and ground glass, the test light may be made to appear as if it were shining through the hole in the mirror or as if it were shining on the illuminated surface of the modifying light Thus the test light would appear either as if it were shining on an unilluminated area or on a coloured surface The object of these experiments was to decide whether stimulation of one retina affected the visibility with the other and, if so, whether the effect was general or limited to corresponding points

Table IV (*a*), V (*a*) and VI (*a*) show the increases in the threshold of the test light when it appears as if shining through the unilluminated centre of the modifying light Table IV (*b*) shows the changes in the threshold of the test lights when appearing as if on the coloured surface of the modifying lights.

In binocular experiments, when attempting to combine the two images so that the test light would appear on the dark area, the direction of the visual axes might unintentionally wander so that the image of the test light would fall outside the fovea. That the visual axes did not move is shown by the constancy of the thresholds for if the image did fall outside the fovea the results would have been irregular and some of the differences in the threshold might have been considerable. In fact, the measurements are practically identical with those yielded by the corresponding appearance obtained when the two lights are shining in the one eye (compare Table I (*a*) with IV (*a*), V (*a*) and VI (*a*).) In the binocular case the result cannot be due to "stray" light.

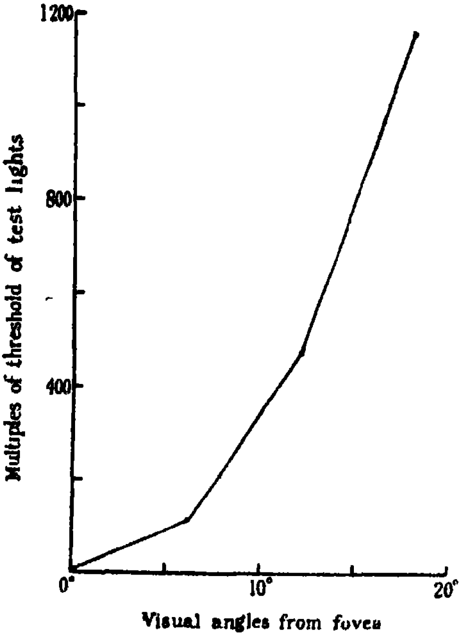


FIG 4.—Variation in effect of surrounding illumination with different distances from the fovea, using a "green" surround and a "blue" test light. Abscissæ, degrees from centre of the fovea. Ordinates, multiples of the threshold of the test light.

The slight raising of the threshold when the light appeared as if shining through the hole in the mirror (that was in fact before the other eye) is not entirely due to narrowing of the pupil, as an artificial pupil was used in some of the experiments. The degree of rise in the threshold does not seem to depend on the colour qualities of the lights.

When the test light appears as if it were shining on the illuminated area

Table IV.—Increase of Threshold of Test Light produced by Binocular Combination for Vision of H E R.

(*a*) Test Light appearing as if on Central Unilluminated Area

Test light.	2.7.30	28.10.30.*	29.10.30*	31.10.30*	5.11.30*	18.3.31*	12.5.31	13.5.31	19.5.31	20.5.31.	22.5.31	27.5.31*	27.5.31.	1.7.31*	Averages			Multiples of the threshold.		
															With pupil	Without pupil	All experiments.	With pupil.	Without pupil.	All experiments.
	mm.	mm.	mm.	mm	mm.	mm	mm.	mm	mm	mm	mm	mm	mm.	mm	mm.	mm.	mm.			
"Red" screen.																				
"Red"	3·8	3·6	3·6	8·6	4·6	9·2	6·2	8·0	16·6	13·0	11·4	13·2	14·0	0·4	6·2	10·3	8·3	2·5	4·6	3·4
"Green"	5·8	1·6	0·8	5·0	6·8	1·8	3·3	4·4	10·0	10·0	10·4	10·2	5·6	1·8	4·0	7·1	5·5	1·8	2·8	2·3
"Blue"	5·4	1·6	4·7	7·0	3·0	3·0	3·5	10·0	8·6	13·2	11·0	0·4	6·8	1·6	3·0	8·3	5·7	1·5	3·4	2·3
"Green" screen.																				
"Red"	4·4	2·4	3·2	8·6	2·2	11·4	6·2	3·0	13·2	10·0	9·6	8·0	14·4	1·2	5·3	8·7	7·0	2·2	3·6	2·8
"Green"	4·4	3·6	1·6	5·2	7·2	1·6	4·3	3·2	8·2	10·4	9·0	9·2	4·6	4·8	4·7	6·3	5·5	2·0	2·5	2·3
"Blue"	5·2	2·0	3·2	9·0	2·6	4·8	3·4	6·8	8·4	12·4	11·4	4·0	7·2	6·8	4·5	7·8	6·2	1·9	3·1	2·5
"Blue" screen.																				
"Red"	2·6	2·2	1·8	4·4	0·8	7·8	5·0	0·9	7·6	8·6	7·6	2·8	6·2	0·2	2·9	5·5	4·2	1·5	2·3	1·8
"Green"	2·4	0·0	1·2	1·2	4·6	−0·2	1·1	2·4	6·4	8·2	7·0	4·0	2·6	−2·0	1·2	4·3	2·8	1·1	1·9	1·4
"Blue"	−2·4	0·2	3·2	7·8	0·4	4·4	0·4	6·2	9·6	8·8	9·4	−2·4	5·2	5·4	2·6	5·3	4·0	1·4	2·2	1·8
Wave-lengths of test lights.																				
"Red"	6478	6552	6279	6304	6429	6678	6503	6527	6853	6353	6371	6779	6508	6629	—	—	—	—	—	—
	6279	6538	6128	6153	6279	6503	6251	6304	6678	6202	6071	6629	6353	6377	—	—	—	—	—	—
"Green"	5397	5397	5324	5422	5275	5397	5575	5726	5701	5575	5677	5677	5677	5551	—	—	—	—	—	—
	5250	5275	5199	5324	5173	5275	5397	5526	5551	5474	5526	5474	5474	5352	—	—	—	—	—	—
"Blue"	4795	4819	4795	4721	4819	4771	4872	4872	4771	4746	4844	4872	4819	4721	—	—	—	—	—	—
	4721	4746	4721	4645	4721	4693	4771	4771	4645	4645	4746	4746	4693	4592	—	—	—	—	—	—

(*b*) Test Light appearing as if on Coloured Background

Test light.	2.7.30	22.10.30*	22.10.30*	4.11.30*	5.11.30*	18.3.31*	12.5.31	13.5.31	19.5.31	20.5.31.	22.5.31	27.5.31*	27.5.31.	1.7.31*	Averages			Multiples of the threshold.		
															With pupil	Without pupil	All experiments.	With pupil.	Without pupil.	All experiments.
"Red" screen																				
"Red"	8·8	9·8	19·2	11·8	—	14·8	8·2	13·0	18·8	15·0	13·8	11·8	15·8	7·4	12·5	13·1	12·8	6·4	6·9	6·6
"Green"	6·8	7·8	16·0	16·3	—	14·4	6·2	6·6	11·[illegible]	13·0	11·8	13·6	11·0	4·0	12·0	9·5	10·7	5·9	4·0	4·2
"Blue"	2·6	19·0	15·8	17·1	—	10·4	9·2	11·6	11·2	13·8	11·4	8·0	11·0	6·0	12·7	10·2	11·3	6·6	4·4	5·8
"Green" screen.																				
"Red"	4·0	2·7	4·6	2·0	—	3·2	6·8	2·8	12·6	16·0	11·4	7·4	9·2	6·0	5·3	8·9	7·3	2·2	3·7	2·9
"Green"	5·7	10·6	10·0	15·0	—	17·2	7·0	7·6	9·0	16·8	11·2	14·4	13·0	5·2	12·1	10·1	11·0	6·0	4·4	5·0
"Blue"	4·4	20·0	15·1	15·2	—	16·8	9·8	12·6	12·8	15·6	12·8	9·6	9·4	4·8	13·6	11·1	12·2	7·4	5·1	6·1
"Blue" screen.																				
"Red"	0·6	2·1	4·0	−0·8	—	6·2	7·3	0·8	6·4	9·6	5·4	5·0	7·4	0·4	2·8	5·4	4·2	1·3	2·3	1·9
"Green"	2·6	4·3	5·2	8·2	—	8·8	7·2	−0·8	3·8	10·4	7·6	5·6	5·0	4·0	6·1	5·1	5·5	2·4	2·1	2·8
"Blue"	4·2	13·0	9·4	12·2	—	12·4	2·6	7·8	8·2	9·0	5·6	4·0	5·6	3·2	9·0	6·2	7·5	3·7	2·5	3·0
Wave-lengths of test lights.																				
"Red"	—	6779	6580	6429	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	6552	6377	6226	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"Green"	—	5525	5449	5324	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	5422	5324	5199	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
"Blue"	—	4921	4844	4710	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	4819	4746	4645	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Artificial pupil used

Table V —Increase of Threshold of Test Light produced by Binocular Superposition on Modifying Light for Foveal and Extra Foveal Vision of H E.R.

Test light	25 11 30	26 11 30	9 11 31	10 11 31	13 11 31	12 11 31	Average	Approximat multiple of threshold
(a) Foveal								
	mm	mm	mm	mm	mm	mm	mm	
" Red " screen								
" Red "	10 4	11 6	9 4	9 6	8 8	11 2	10 2	4 5
" Green "	14 0	14 0	9 6	9 8	10 2	12 4	11 7	5 7
" Blue "	10 2	9 4	13 6	7 8	9 6	12 5	10 5	4 7
" Green " screen								
" Red "	4 6	3 4	8 2	10 0	3 8	10 4	6 7	2 7
" Green "	11 2	13 6	9 6	12 2	9 6	14 0	11 7	5 7
" Blue "	10 0	7 4	14 4	9 4	9 4	11 3	10 3	4·6
" Blue " screen								
" Red "	1 6	2 0	3 8	6 0	1 2	4 2	3 1	1 5
" Green "	1 6	3 8	3 0	5 0	2 0	6 4	3 9	1 7
" Blue "	6 2	0 6	10 4	1 6	4 8	5 9	5 1	2 1
(b) Extra Foveal (21°)								
" Red " screen								
" Red '	2 6	5 2	5 6	10 9	11 8	13 6	8 3	3 4
" Green '	10 6	15 4	9 2	12 8	13 2	8 8	11 7	5 7
" Blue "	11 2	11 4	17 2	14 0	9 6	7 0	11 7	5 7
" Green " screen								
" Red "	4 0	6 6	10 4	14 4	12 4	13 6	10 2	4 5
" Green "	11 6	14 6	11 2	14 0	11 4	11 0	12 3	6·2
" Blue "	9 4	14 8	18 8	15 0	15 4	18 4	15 3	9 4
" Blue " screen								
" Red "	1 0	5 8	9 0	13 4	13 8	9 6	8 7	3 6
" Green "	7 0	4 5	9 0	15 2	10 6	6 2	8 7	3 6
" Blue "	6 8	12 2	15 2	13 6	10 6	11 6	11 7	5 7
Wave lengths of test lights								
" Red "	6328	6377	6678	6779	6629	6655	—	—
	6153	6177	6503	6655	6429	6429	—	—
" Green "	5449	5495	5449	5551	5397	5575	—	—
	5275	5352	5299	5324	5275	5397	—	—
" Blue "	4746	4669	4645	4844	4872	4771	—	—
	4623	4623	4543	4721	4746	4669	—	—

Table VI —Increase in Threshold of Test Light Produced by Binocular Superposition on Modifying Light for Foveal and Extra Foveal Vision of H E R

Test lights	4 11 31	6 11 31	Average	Approximate multiple of threshold
		(a) Foveal		
	mm	mm	mm	
"Red" screen				
"Red"	12 6	12 8	12 7	6 5
"Green"	8 2	12 8	10 5	4 7
"Blue"	11 0	9 8	10 4	4 7
"Green" screen				
"Red"	9 6	6 8	8 2	3 3
"Green"	9 8	9 6	9 7	4 2
"Blue"	13 4	7 8	10 5	4 7
"Blue" screen				
"Red"	3 0	5 0	4 0	1 8
"Green"	3 0	5 8	4 4	1 9
"Blue"	10 2	5 2	7 7	3 1
		(b) Extra Foveal (21°)		
"Red" screen				
"Red"	13 6	14 6	14 1	8 0
"Green"	35 8	24 1	30 0	83 2
"Blue"	38 4	40 4	39 4	325 0
"Green" screen				
"Red"	18 4	10 6	14 5	8 5
"Green"	38 0	25 6	31 2	109 0
"Blue"	38 8	48 2	43 5	610 0
"Blue" screen				
"Red"	11 4	12 2	11 8	5 7
"Green"	31 8	16 9	14 3	36 2
"Blue"	38 8	51 6	45 2	780 0
Wave-lengths of test lights				
"Red"	6678	6678		—
	6503	6478	-	—
"Green"	5578	5650	--	—
	5449	5474	-	—
"Blue"	4669	4771	-	—
	4543	4645	—	—

there is a greater increase in the thresholds and the quality of the light does then seem to have some influence The "red" modifying light seems to raise the thresholds of all the test lights to about the same extent; the "green" modifying light raises the thresholds for the "green" and "blue" test lights more than it raises the threshold of the "red" test light, and the "blue" modifying light causes a progressive increase in the thresholds of the test lights from the long to the short wave-lengths

A few measurements were made using regions of the retina outside the fovea It was not possible to make measurements when the test light appeared as if shining through the hole in the mirror because it was too difficult to make the two fields superimpose correctly Therefore only observations are recorded when the test light appeared as if on the coloured illuminated surface (see Tables V (*a*) and VI (*a*))

PART II —SIMULTANEOUS ILLUMINATION OF THE SAME RETINAL AREA

Experimental Methods

Two methods were used For the first method the optical arrangement used was that described in the previous paper (Roaf, 1928), except that instead of two illuminated areas being shown side by side on the ground glass screen and then superimposed, one large illuminated area and one small test area were used, which could be arranged in superposition The optical arrangement is shown in fig 5 The image of the disc aperture in diaphragm A, on which the spectrum is formed, is projected on the ground glass screen E by means of the prism (P) and lens (L) At the same time the lens (L) forms a large patch of colour from the colour filter on the same ground glass (E) The diaphragm at A and the ground glass screen at E occupy conjugate planes and the opal lamp in the opaque box and the ground glass screen E also are situated in conjugate planes

By this system it is possible to measure the threshold of the light passing through A by itself, using a photometric wedge in front of A and remeasure the threshold when the light in the opaque box is turned on By means of the shutter the intensity of the modifying light could be varied

For the second method a spectroscope with two collimators was used (Roaf, 1927, p. 381) One collimator had the longest slit that could be fitted to it. The other had a short slit about one-third the length of the other. These collimators were so arranged that the spectrum from the smaller slit occupied the centre of the other one On looking through the eyepiece a slit-shaped aperture was visible which was illuminated by three patches of light, the upper and lower being produced by one spectrum and the middle by the superposition of both spectra The shorter patch is the test light and the longer one the modifying light By means of photometric wedges the threshold for either collimator could be determined separately and the thresholds redetermined when both collimators were transmitting light

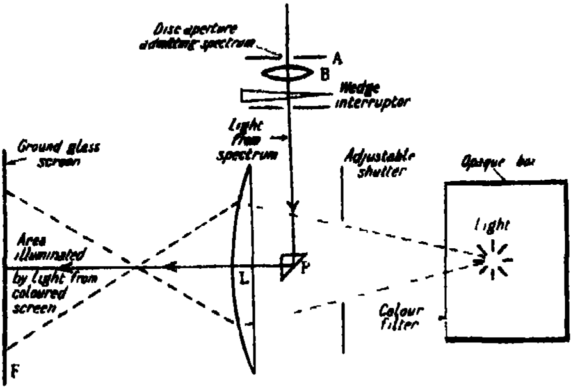


Fig 5—Arrangements for superposition of test light on a large area of modifying light The light in the opaque box is focussed by the large lens (L) on the ground glass screen (E) forming a large illuminated area of the colour of the colour filter The intensity of the modifying light can be altered by the adjustable shutter The light from the disc aperture in the spectroscopic lantern is focussed by the small lens (B) and the large lens (L) with the intermediation of the totally reflecting prism (P) on the ground glass screen (E) The ground glass screen (E) is at a conjugate plane to the diaphragm (A) and also to the light in the opaque box The observer seated beyond the ground glass screen sees a small image of the disc aperture on the large coloured background The spot of light can be interrupted by a slowly revolving sectored disc This apparatus without the totally reflecting prism and the optical parts to the side was used for the modifying light in fig 1 The large ground glass screen (E) in that figure represents the ground glass screen in this one

In some of the experiments the light from the shorter slit was interrupted by a slowly rotating sector for the same reason as that given on p 451 The figures plotted exhibit the differences between the threshold of the test light seen alone and when superimposed upon the modifying light Each measurement of the threshold is the average of five separate readings

Results

The results are shown by a series of curves and they agree with those described in a previous paper (Roaf, 1928). Figs 6 to 11 show the effect of a large illuminated area, at different intensities on the threshold of the test lights for foveal

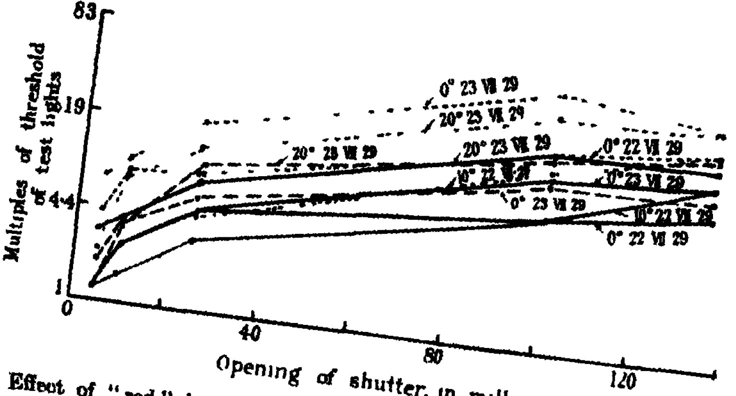


FIG 6 – Effect of "red" background on differential thresholds for foveal and extra foveal vision of right eye of H E R Abscissæ, opening of shutter in millimetres, full opening being 135 mm , the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test light

"red" test light, 6429–6202 Å on 22 7 29, and 6629–6454 Å on 23 7 29

"green" test light, 5474–5324 Å on 23 7 29

"blue" test light, 4893–4819 Å on 22 7 29, and 4771–4721 Å on 23 7 29

The degree of eccentricity and date of experiment is marked against each curve

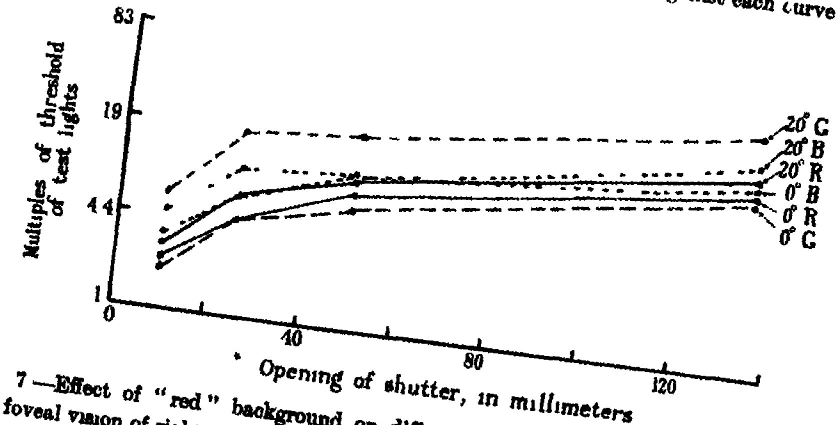


FIG 7 —Effect of "red" background on differential thresholds for foveal and extra foveal vision of right eye of H.E R Abscissæ, opening of the shutter in millimetres, full opening being 135 mm , the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test lights

———— "red" test light, 6377–6177 Å on 3 10 30

— — — "green" test light, 5551–5474 Å. on 7 10 30

· "blue" test light, 4819–4771 Å. on 7 10 30.

The degree of eccentricity is marked against each curve.

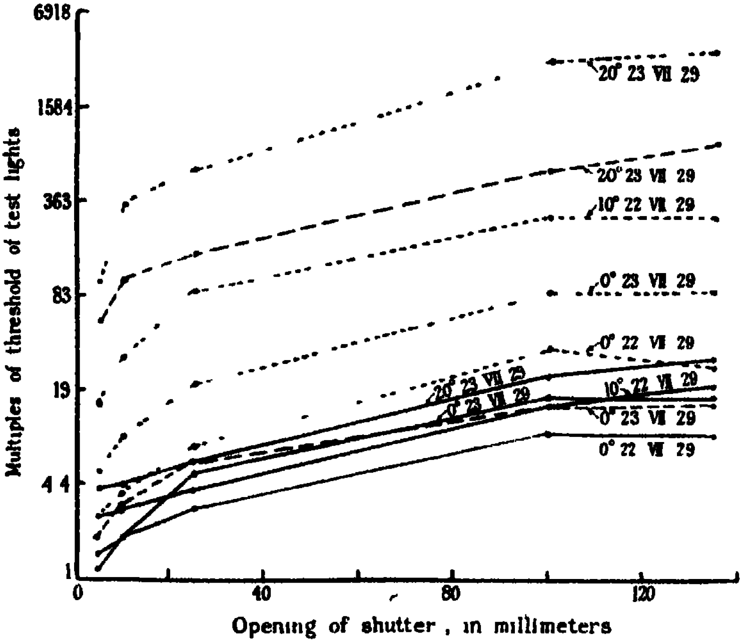


FIG 8—Effect of "green" background on differential thresholds for foveal and extra foveal vision of right eye of H E R Abscissæ, opening of shutter in millimetres, full opening being 135 mm, the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test light

———— "red" test light, 6429–6202 Å on 22 7 29, and 6629–6454 Å on 23 7 29

- - — "green" test light, 5474–5324 Å on 23 7 29

"blue" test light, 4893 4819 Å. on 22 7 29, and 4771–4721 Å on 23 7 29

The degree of eccentricity and date of experiment is marked against each curve

and for extra foveal vision The "red" background raised the threshold for all the test lights to about the same extent for both foveal and extra foveal vision The "green" background raised the foveal thresholds for all the test lights to about the same extent as did the "red" background The threshold outside the fovea for the "red" test light was raised to about the same extent as the foveal thresholds for all the test lights, but the thresholds outside the fovea for "green" and for "blue" were raised to a considerable extent The "blue" background raised the foveal thresholds for the three test lights to a less degree than did the "red" and "green" backgrounds The "blue" background likewise raised the threshold outside the fovea for "red" but slightly. On the other hand, the "blue" background raised the thresholds outside the fovea for the "green" and "blue" test lights to a considerable degree

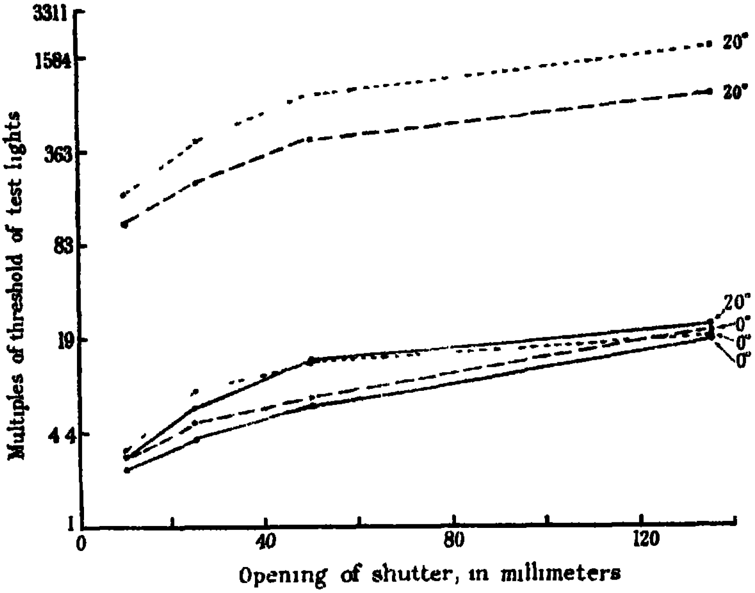


Fig 9—Effect of "green" background on differential thresholds for foveal and extra foveal vision of right eye of H E R Abscissæ, opening of the shutter in millimetres, full opening being 135 mm , the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test lights

——————-- "red" test light, 6377–6177 Å. on 3 10 30

— — -- - "green" test light, 5551–5474 Å. on 7 10 30

"blue" test light, 4810–4771 Å on 7 10 30

The degree of eccentricity is marked against each curve

With the spectroscope fitted with two collimators the observations were confined to foveal vision Figs 12, 13 and 14 show the results on three individuals using different intensities of the background or modifying light. It is seen that the "green" and "blue" modifying lights have least effect on the thresholds for "red," and the readings actually show, with the weaker intensities, a fall in the threshold for "red," but these differences are too small to indicate definitely an actual increase in sensitivity, to the "red" test light The "red" background raised the threshold of test lights of all colours more than did either the "green" background or the "blue" background.

Fig 15 shows the extent to which equal multiples of the threshold of the modifying lights raise the thresholds of test lights of different wave-lengths. Fig 16 shows corresponding curves for another observer. There are two

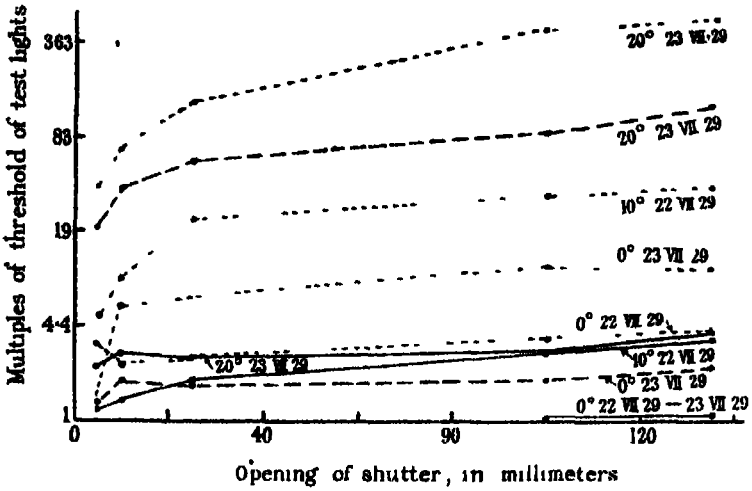


FIG 10—Effect of "blue" background on differential thresholds for foveal and extra foveal vision of right eye of H E R Abscissæ, opening of shutter in millimetres, full opening being 135 mm, the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test light.

———— "red" test light, 6429–6202 Å on 22 7 29, and 6629–6454 Å on 23 7 29

— — — — "green" test light, 5474–5324 Å on 23 7 29

. . . . "blue" test light, 4893–4819 Å on 22 7 29, and 4771–4721 Å on 23 7 29

The degree of eccentricity and date of experiment is marked against each curve

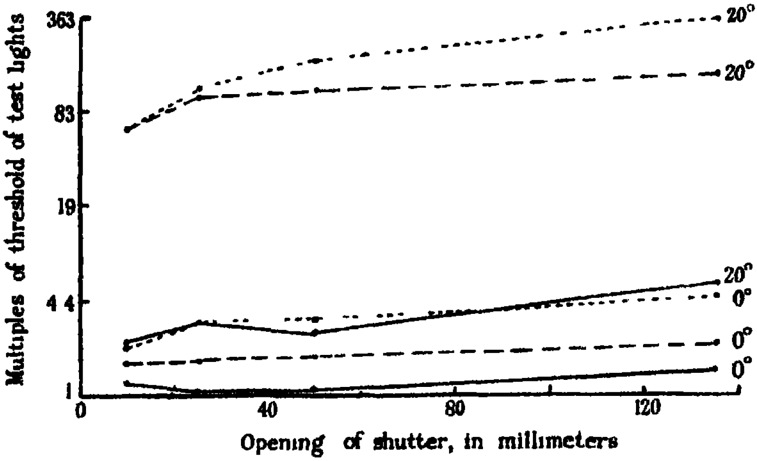


FIG 11—Effect of "blue" background on differential thresholds for foveal and extra foveal vision of right eye of H E R Abscissæ, opening of the shutter in millimetres, full opening being 135 mm, the readings being proportional to the intensity of the modifying light Ordinates, multiples of the threshold of the test lights.

———— "red" test light, 6377–6177 Å on 3 10 30

— — — — "green" test light, 5551–5474 Å on 7.10.30

. .. "blue" test light, 4819–4771 Å on 7 10 30

The degree of eccentricity is marked against each curve outstanding features of these curves Firstly, the curves in fig 15 tend to be horizontal at the long wave-length end of the spectrum, but the measurements of fig 16 were not taken at sufficiently long wave-lengths to show the horizontal portions This indicates that for any one background the effect on the "red" test light does not vary with the actual wave-length of the "red"

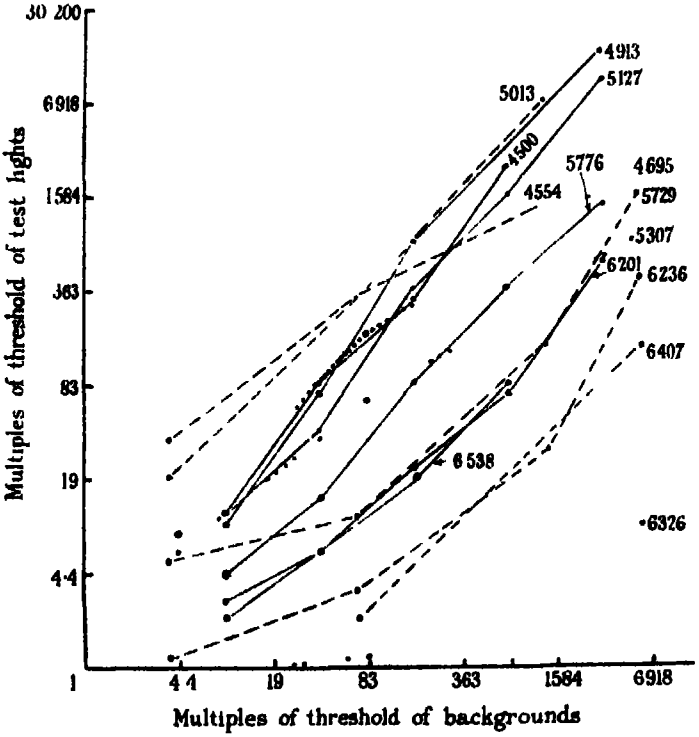


FIG 12—Effect of different intensities of various backgrounds on differential thresholds of H E R Abscissæ, multiples of the threshold of the background Ordinates, multiples of the threshold of the test lights

——— ——— "red" background, 6214 Å

— — — "green" background, 5404 Å

. "blue" background, 4708 Å

Wave-length of test light marked against each curve

provided that it has a wave-length longer than about 6200 Å. With test lights from other parts of the spectrum a slight change in wave-length of the test light makes a considerable difference to the effect on the threshold. In other words, the "red" of the spectrum varies only in intensity but not in quality, in fact it is "physiologically monochromatic" This peculiarity was noted previously (Roaf, 1930), and fig 18 is another example which is inserted here to show that when a number of measurements are made at the "red" end of the spectrum there is no tendency for the curve to have any inclination in that region In the experiment from which fig 18 was drawn there was no attempt to equalise the intensities of the backgrounds

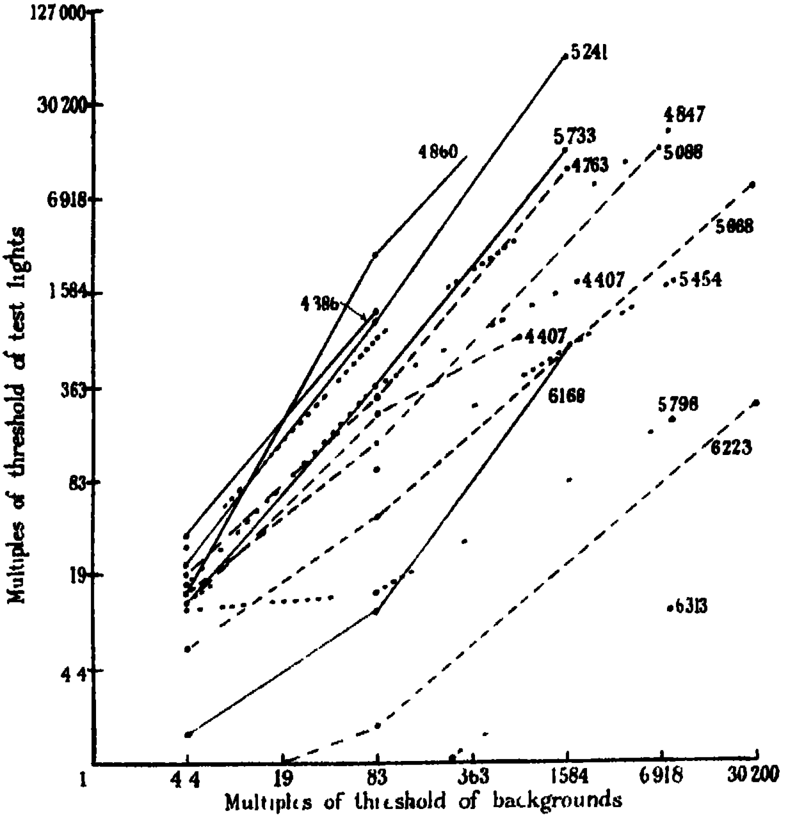


FIG 13 —Effect of different intensities of various backgrounds on differential thresholds of D R Abscissæ, multiples of threshold of backgrounds Ordinates, multiples of threshold of test lights

- — - – " red " background, 6214 Å.
- – — " green " background, 5404 Å
" blue " background, 4708 Å

Wave-length of test light marked against each curve

This behaviour of lights of wave-lengths longer than 6500 Å has been noted by others, but it is surprising that it extends so far into the " orange " as is shown in some of the curves It may be that the experimental method employed does not lend itself to show small differences at that region of the spectrum.

Secondly, the effect of any background is always greater on the shorter wave-lengths than on the "red" test lights. This can be correlated with the great decrease in sensitivity to the shorter wave-lengths on adaptation to light.

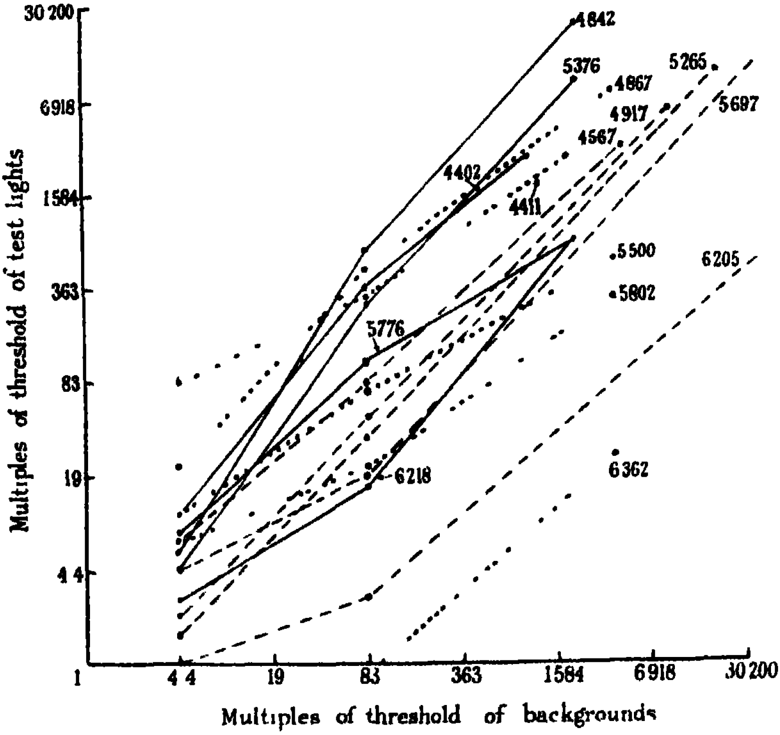


FIG 14.—Effect of different intensities of various backgrounds on differential thresholds of R M Abscissæ, multiples of threshold of backgrounds Ordinates, multiples of threshold of test lights

———— "red" background, 6214 Å
— — — — "green" background, 5404 Å.
"blue" background, 4708 Å

Wave length of test light marked against each curve

On account of the different law relating brightness to intensity at different wave-lengths another comparison was made Three regions of the spectrum were compared by the "equality of brightness" method and the multiples of their thresholds measured when they appeared of equal brightness. Fig 17 shows the result of an experiment in which the modifying lights were such multiples of their respective thresholds that they would have appeared of equal brightness

The curves shown in figs. 15 and 16 correspond to the values given by the curves in figs 12, 13 and 14 where they cut vertical lines drawn from the axis of the abscissæ for a value of 83 times the threshold The curves shown in fig 17 correspond to the values given by the curves in figs 12, 13 and 14 where they cut vertical lines drawn from the axis of the abscissæ for values of 83, 363 and 4467 times their respective thresholds for "red," "green" and "blue" backgrounds respectively That the values recorded in figs 15, 16 and 17 are of about the right order indicates that the values are approximately correct

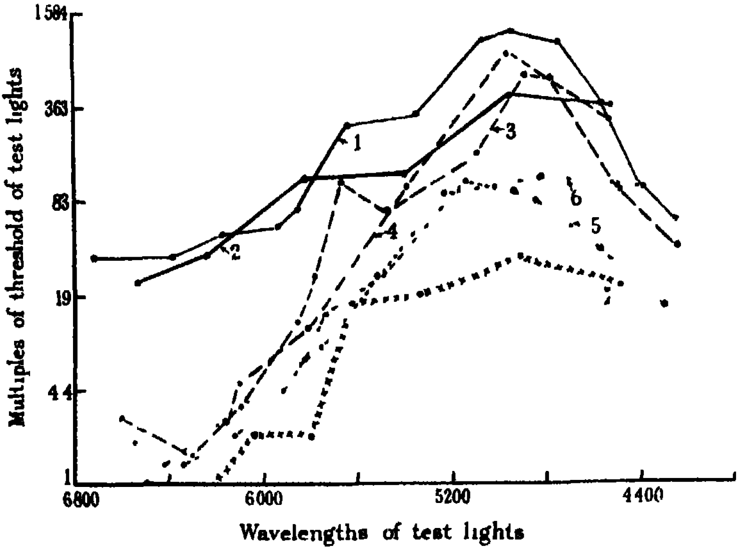


FIG 15—Effect of equal multiples of the threshold of the background on differential threshold of H E R Abscissæ, wave lengths of test lights Ordinates, multiples of the threshold of test lights

——— "red" background, 1, 6428 Å. on 5 3 31, and 2, 6300 Å on 27 10 31
— — — — "green" background, 3, 5404 Å. on 10 3 31, and 4, 5274 Å on 27 10.31
. "blue" background, 5, 4797 Å. on 11 3 31, and 6, 4695 Å. on 28 10 31
× × × × "violet" background, 7, 4487 Å on 17 3 31

Discussion

The results indicate that the thresholds of the test lights are markedly influenced by two factors

For foveal vision the thresholds are raised mainly as the result of simultaneous exposure of the same area of the retina.

For vision outside the fovea a neighbouring area of illumination can cause a considerable increase of the threshold, possibly due to interconnections in the layers of the retina

Foveal Vision with One Eye.

Evidence has been obtained that the influence of neighbouring regions is negligible for foveal vision (Tables I (*a*) and II (*a*)) and that the decrease in illumination of the retina due to narrowing of the pupil is small It must be pointed out that the test light and modifying light were not in contact, but in one experiment in which they were brought into contact a similar result was obtained The closer the two lights the greater will be the overlap of illumination due to spherical aberration and other optical processes Therefore it must be understood that the spread of effect is negligible over a distance represented by a visual angle of about 1°

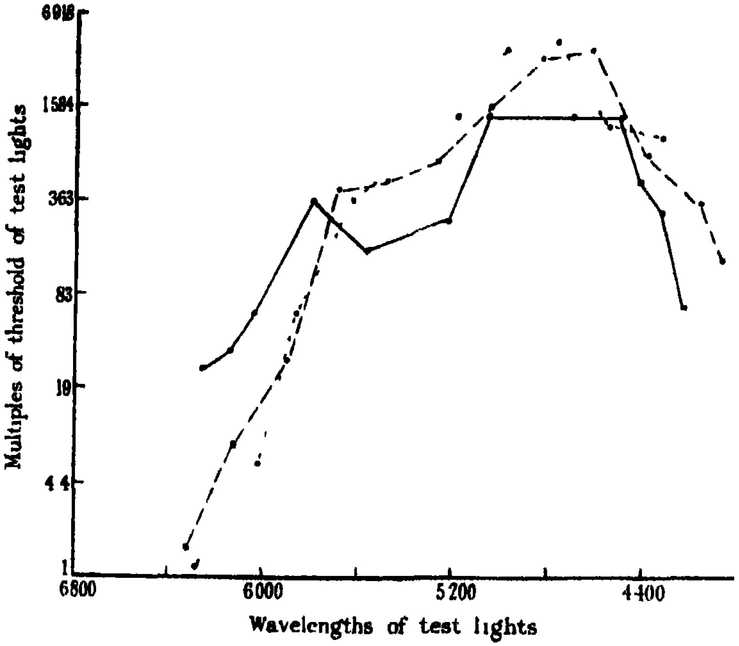


FIG 16—Effect of equal multiples of the threshold of the background on differential thresholds of H F Abscissæ, wave lengths of test lights Ordinates, multiples of threshold of test lights

--- - "red" background, 6300 Å on 24 6.31
– "green" background, 5404 Å on 26 6 31
"blue" background, 4708 Å on 30 6 31

The effect of superposition agrees with that previously described (Roaf, 1928) The "red" background raises the threshold for the whole spectrum whilst the "green" and "blue" backgrounds have comparatively little effect on the long wave-length end of the spectrum These effects were investigated from several points of view First of all, the thresholds in the dark might not be correct A fixation point was not used when observing through the spectroscope The precautions taken to measure the thresholds are described above (p 452) The experiments recorded in figs 12, 13 and 14 were carried out in order to control the values of the absolute thresholds If, owing to inaccurate fixation, the absolute thresholds were too low, fixation of the direction of vision by the weakest modifying light would cause a great increase in the threshold, but there would be relatively less difference with the higher intensities of modifying lights Many of the curves are approximately straight lines of which the formula is

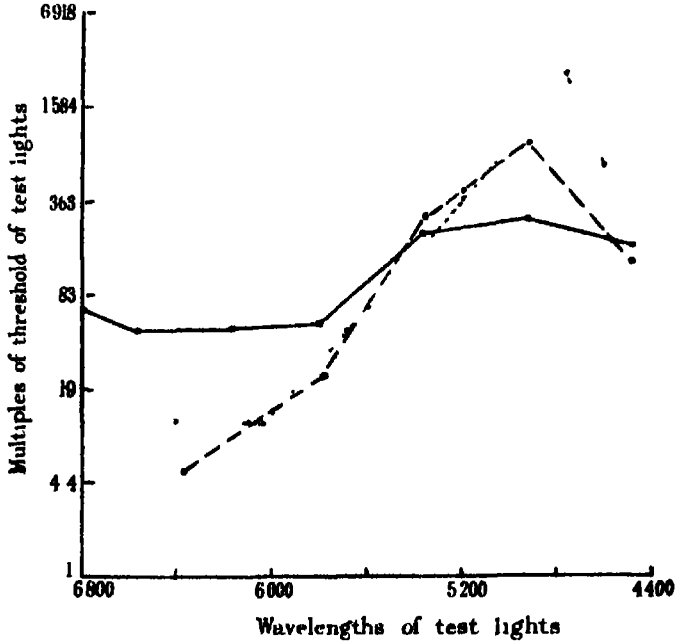


FIG 17 —Effect of equal brightnesses of background of various wave-lengths on differential thresholds of H E R Abscissæ, wave lengths of test lights Ordinates, multiples of threshold of test lights

———— "red" background, 6300 Å, 83 times the threshold on 4 11 31
— — — — "green" background, 5291 Å. at 363 times the threshold on 30 10 31
"blue" background, 4708 Å., 4467 times the threshold on 2 11 31

Log intensity of test light = k × log intensity of modifying light + a constant

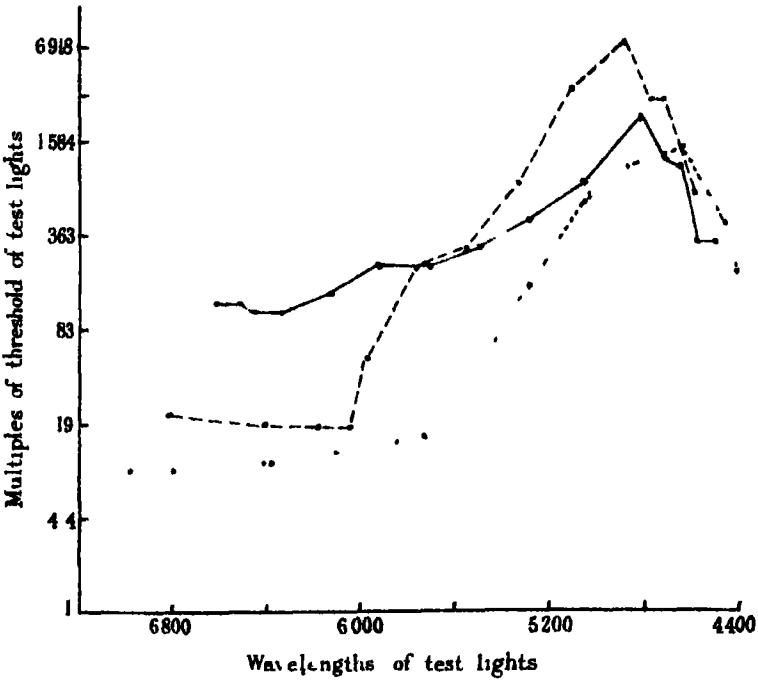


Fig. 18.—Effect of unequal intensities of background of various wave lengths on differential thresholds of H E R Abscissæ, wave-lengths of test lights Ordinates, multiples of the threshold of test lights

———— "red" background, 6214 Å on 17 6 30

— — —— "green" background, 5404 Å on 17.6 30

"blue" background, 4708 Å on 18 6 30

If one calculates the values of *k* from the extreme values of the curves the following values are obtained —

	Fig 11	Fig 12	Fig 13	Average
"Red" background	1 012	1 270	0 997	1 093
"Green" background	0 836	0 873	0·908	0 872
"Blue" background	0 576	0 758	0 659	0 698

The above values are only approximate as some of the curves are not straight lines. Further (see discussion later, in connection with figs 6 to 11), it is probable that the curves do bend at the lower values The values of *k* are much greater than one would expect for the Weber-Fechner Law as determined for higher intensities and with mixed white light. This difference suggests that the curves bend at higher values It is curious that the Weber-Fechner Law has not been investigated previously with light from limited regions of the spectrum Extrapolating from all the curves towards the base line, it is found that the average value would be practically at the junction of the axis of the abscissa line with the axis of the ordinates The curves for the "red" test lights are probably concave whilst whose for the "green" and "blue" test lights may be convex

Table VII —Decrease in Threshold for "Red" Test Light when Superimposed on "Green" and on "Blue" Modifying Lights for Right Eye of H E.R

Fig	Background		Test light		
	Wave-lengths A	Multiple of threshold	Wave lengths A	mm	Multiples of threshold
9	4708	4 4	6326	—1	1/1 1
10	5404	4 4	6223	—5	1/2 1
10	4708	4 4	6313	—8	1/3 2
10	4708	83 2	6313	—5	1/2 1
11	4708	4 4	6362	—5	1/2 1
11	4708	83 2	6362	—1	1/1 1

In figs 12, 13 and 14 several curves start from the base line some distance to the right of the origin That is because these curves showed an actual increase in sensitivity with the weaker illuminations. These figures for the increase in sensitivity are tabulated above (Table VII) The differences are small and might be due to summation of subminimal values of the test lights with "stray" light, but they show that even if the "green" and "blue" backgrounds do not reinforce the "red" test light they do not interfere appreciably with its visibility at the lower intensities of the backgrounds This suggestion of a decrease in the threshold for "red" with "green" and "blue" backgrounds is of the same nature as that discussed in the preceding paragraph, namely, that these curves are concave.

In addition to the difficulty discussed above in relation to the measurements of the absolute thresholds a second difficulty presented itself, namely, how to compare the intensities of lights of different colour with one another The energy value of the light cannot be used because the brightness for the same energy value is not the same for different wave-lengths. Even if one uses an arbitrary comparison, such as the "flicker" method, the two brightnesses will no longer be equal if the energy of each is equally reduced or increased because of the Purkinje phenomenon Two methods of comparison have been used, namely, to make measurements of the backgrounds at equal multiples of the threshold and at equal brightnesses

Figs. 15 and 16 show that when the backgrounds are equal multiples of their respective thresholds, the thresholds of the " red " test lights are raised much more by the " red " than by the " green " or " blue " backgrounds

A measurement was made by the " equality of brightness " method of the multiples of the threshold when the lights appeared of equal brightness This measurement was carried out by the arrangement of the spectroscope as used for the measurement of discrimination (Roaf, 1927)

A light of 6300 Å 83 times the threshold appeared equally bright as one of 5291 Å 325 times the threshold, and also equally bright as one of 4708 Å 3802 times the threshold Fig 17 shows the result with backgrounds of 6300, 5291 and 4708 Å at 83, 363 and 4467 times the threshold respectively Once more one sees that the threshold of the " red " test light is raised less by the " green " and " blue " backgrounds than by the " red " one

The " red " of the spectrum shows another characteristic feature When different wave-lengths of " red " are used as test lights it is found that for any one background the threshold is equally raised This equal effect of the background on the different test lights suggests that whatever receptors are stimulated they are stimulated in the same ratio, but it does not necessarily imply that only one type of receptor is stimulated. This uniformity of the " red " of the spectrum, namely, that it does not differ in quality but only in brightness can be expressed by saying that it is " physiologically monochromatic " Fig 18 shows this phenomenon by the almost horizontal lie of the curves at the long wave-length end of the spectrum

With all the backgrounds in figs 12 to 18 it is seen that the thresholds of the shorter wave-lengths are raised to a greater degree than those for the longer ones This greater increase in the threshold must be related to the greater change in sensitivity of the former than of the latter during adaptation Thus any light shining on the retina raises the threshold for " green " and " blue " There is a peak in the curves about 4900 Å. which suggests that adaptation for " blue " is less than for " green " There is a possibility that the " violet " of the spectrum contains a " red " element, hence the decreased effect in increasing the threshold

A curve showing similar low values at the extremes of the spectrum is given by Hecht (1931, (i) and (ii)) from the results of Priest and Brickwede (1926) The latter do not give curves, hence Hecht must have obtained his curves from unpublished data The maximum of the curve given by Hecht does not correspond in wave-length with the maximum figs 15, 16, 17 and 18, so it is doubtful whether there is any relation between these results and those of Priest and Brickwede

Vision Outside the Fovea with One Eye

Tables I (*b*) and II (*b*) show the marked action of a neighbouring field of illumination on the thresholds of the test lights The thresholds of "green" and "blue" are raised more than that for "red," which is probably related to the greater degree of adaptation of the peripheral retina for short wave-lengths

The long wave-lengths show an individuality, namely, that they do not raise the thresholds to the other lights, nor are they affected by the other lights to a marked degree This individuality is shown in two other ways namely, the less effect of the shorter wave-lengths on "red" described in the previous section and the relatively low sensitivity to "red" of the peripheral retina

It is not easy to decide how neighbouring illumination raises the threshold for the test lights The refraction of eccentric rays may differ from those falling on the fovea, thus images may not be so sharp and some of the effect may be due to "scattering" with consequent superposition of light. In view of the magnitude of the effect and the different way in which the quality of the light affects vision outside the foveal, as contrasted with foveal vision, it does not seem possible that the whole increase in threshold is due to imperfect formation of images on the retina outside the fovea

Ramón y Cajal has shown that there are lateral connections between different parts of the retina, and Adrian and Matthews (1928) have given experimental evidence for interconnections in the eel's eye

Granit and his co-workers (1930, 1931) have shown that with multiple rotating discs the interval for extinction of "flicker" is less with several discs than with a single one They ascribe this difference to "synaptic convergence" It is difficult to know how much of the difference is due to cross illumination from one disc to the image of the other, thus actually increasing the intensity of illumination with consequent decrease in the interval for abolition of "flicker." This scattering of light was discussed in a previous paragraph. If the increases in threshold on the retina outside the fovea are not due to imperfect formation of images the results show more striking evidence than that of Granit for "synaptic convergence" either in the retina or centrally. In a paper on the influence of wave-length on acuity of vision it was pointed out that the poor acuity in "blue" light might be due to a number of receptors being linked to a single ganglion cell (Roaf, 1930 (i)).

Whatever the explanation may be, the increase in threshold due to neighbouring illumination must be an important factor in the adaptation to light (induction) of the retina outside the fovea.

Superposition of lights on the portions of the retina other than the fovea produces decrease in sensitivity comparable to that shown by neighbouring illumination Figs 6 to 11 show curves in which it is seen that the thresholds outside the fovea are not raised by a "red" background to as great a degree as those of the "green" and "blue" test lights by the "green" and "blue" backgrounds As in figs 12 to 14 the measurements were made with different intensities of background The bend in the curves with the lowest illuminations, figs 6, 8 and 10, indicates that if the measurements could have been made with still lower intensities of background they would have commenced at the absolute threshold Further, the same absolute thresholds were used for the "red" backgrounds, figs 6 and 7, therefore the high values in figs 8 to 11 cannot have been due to misreadings of the absolute thresholds As in the section on foveal vision, it seems that the absolute thresholds are not greatly at fault It is impossible to state how much of the increase in threshold is due to neighbouring illumination and how much to superposition of the lights

Although the light transmitted by the "blue" screen, as judged by the fovea, is a lower multiple of its threshold than that transmitted by the "red" and "green" screens, the magnitude of the effect on the retina outside the fovea is remarkable, figs 10 and 11 This result shows how impossible it is to apply the same standard of intensity to the foveal retina and to parts outside of it

Binocular Vision

The increases in threshold due to binocular combination of the test light with the modifying light, so that the test light appears as if shining through the hole in the mirror, are small, and of the same order as those due to surrounding illumination in foveal uniocular vision These differences cannot be due to retinal interaction but must be due to central processes. Is it possible that the similar effect in uniocular vision is partly due to central processes?

When the binocular combination is arranged so that the test light appears as if on the illuminated surface the differences in the threshold are greater than when the light appears as if on the unilluminated area The increases are what one would expect if the hypothesis of colour vision given elsewhere

(Roaf, 1930 (ii)) were correct, namely, that there are three sets of receptors, one sensitive to the whole of the spectrum, a second sensitive to "red" and "green," and a third stimulated only by "red", thus "red" light stimulates all three sets, "green" stimulates two, and "blue" stimulates one

If "red" light stimulates all three sets of receptors, the red sensation must be accompanied by inhibition of nerve impulses that would give rise to sensations of green and blue This inhibition must not be too great, otherwise mixture of colours would be interfered with It is seen, Table IV (*b*), that the "red" background has raised the threshold for all three test lights If "green" light stimulates the receptors for the whole spectrum as well as those sensitive from 4900 Å to the extreme "red," then stimulation by it will give rise to inhibition of impulses that would otherwise produce the sensation of blue It is seen in Table IV (*b*) that the "green" modifying light raises the thresholds for "green" and for 'blue" more than for ' red" If "blue" stimulates only the receptors for the whole spectrum it will have less effect on the thresholds for "red" and for ' green" than for "blue" It is seen that the thresholds are raised progressively from the long to the short wave-lengths by the "blue" modifying light

These differences are not very large and, as pointed out above, one would not expect them to be great because then recognition of colour mixtures would be impossible Attention is drawn to them here to show that the results are not contradictory to the hypothesis that "red" stimulates three types of receptors, "green" stimulates two, and "blue" stimulates one Of course with high intensities the limitation is not so specific and a sufficiently bright "blue" might stimulate all receptors to a certain extent with the result that the purity of the light would suffer, it being mixed with white

It was not found possible to measure binocular vision outside the fovea with the test light apparently on the unilluminated area, but some measurements were made with the test light appearing as if it were on the coloured background, Table V In most of the measurements the increase in threshold was not much greater than with foveal binocular vision, therefore suggesting that the uniocular effect outside the fovea is due to some retinal interference and not to central processes In two experiments a result similar to that found with uniocular vision outside the fovea was obtained, Table VI It may be that the results were due to rivalry or that the diffuseness of localisation in the retina outside the fovea is also represented, to some extent, in the central nervous system. Unfortunately, no method has been evolved to decide this point. As shown in Table V, repetitions of these experiments gave results agreeing with those for foveal binocular vision, Table IV (*b*) On the other hand, the results shown in Table VI may be due to some undiscovered error in the experiments

The subject of central inhibition of impulses which would give rise to colour sensations, requires further examination There are many observations in binocular vision, such as the Fechner effect, which show that either occlusion or inhibition is taking place Hartridge (1915) shows an interesting example of inhibition of visual impulses (experiment 4, p 48)

Allen and his co-workers have published many papers on the interaction of retinal areas which they describe as "visual sensory reflexes" They have found that illumination of one part of the retina can affect another part either of the same or of the opposite eye The observations were made by measuring the duration of the interval of interruption of light when a steady sensation was produced The line of argument seems to be that since the duration of interruption must be shorter when the intensity of light is increased, therefore, if the intensity is kept constant, and the duration of interruption is diminished, the light is then equivalent to a brighter light, or, in other words, the sensitivity of the retina is increased It is doubtful whether sensitivity measured by such a method will correspond to sensitivity as measured by the threshold intensity of light. Allen finds that the sensitivity of the opposite eye may be either increased or diminished depending upon the intensity of the modifying light According to the results described in the present investigation there is always a decrease in sensitivity of the opposite eye, that is, the intensities correspond to the α-intensities of Allen (1923, 1926)

It is well known that the sensitivity of the retina increases during dark adaptation, but according to Allen (1923, 1926, 1931) exposure to coloured lights of certain intensities increases the sensitivity Therefore it is clear that the processes measured are not the same in both cases Allen measured differences at higher intensities and by the flicker method, whilst dark adaptation is measured by the decrease in the threshold for light.

Incidental Observations

When using a "blue" test light for foveal vision there is sometimes a sensation of pulsation. This is most marked when the threshold is considerably raised, *e g* , by a "red" modifying light The sensation seems to be due to the rhythmical illumination of the retina outside the fovea, where the sensitivity for blue is much greater than on the fovea, by light "scattered" by the optical surfaces and eye media One must be careful not to confuse this sensation of the whole eye being illuminated with the interruption of the test light as seen by the fovea

The effect of "scattering" of light was frequently observed by the appearance of a coloured veil, of the same colour as the modifying light, over the supposedly unilluminated area, as well as by the experiment of superimposing the test light on the blind spot It is the presence of this "scattered" light which makes it impossible to decide how much of the observed effect is actually due to superposition of light and how much to interference between nerve impulses acting on the same cell

One striking feature throughout the investigation was the closer relationship between "green" and "blue" than between either of these colours and "red" This similarity is seen also in the sensitivity of the extra foveal retina, in the adaptation to light and in the photochromatic interval

Summary

(1) For foveal vision with one eye, the differential threshold for visual sensations is markedly affected by simultaneous illumination of the same area of the retina The quality of the light has a great influence on the magnitude of the difference The long wave-lengths raise the threshold for the whole spectrum The shorter wave-lengths raise the threshold of the shorter wave-lengths but have comparatively little effect on the long ones Change in sensitivity due to the modifying light is the phenomenon known as adaptation to light, sometimes known as temporal induction

(2) For vision outside the fovea with one eye, illumination of a neighbouring region of the retina can raise the threshold either by overlapping of the images or by interference with the conduction in the nerve paths of the retina or central nervous system Lights of various colour have different modifying effects outside the fovea from those which they show inside it since the "green" and "blue" have a greater effect than the "red" outside the fovea Spread of effect from one part of the retina to another is a factor in what is known as spatial induction

(3) With binocular vision there is a slight increase in the threshold of one eye due to stimulation of the other, thus indicating central inhibition of nerve impulses

(4) Some minor points arising out of the investigation are discussed

I have much pleasure in thanking the Trustees of the London Hospital Research Fund for a grant from which the expenses of this research have been

defrayed I have also much pleasure in acknowledging the help of the various subjects for making the somewhat arduous observations.

To Sir Charles S Sherrington I am much indebted for his interest and help in the writing of this paper

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The Rhythmic Discharge of Motoneurones

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[Plates 24 and 25.]

I Introduction

Denny-Brown (1929) and Adrian and Bronk (1929) have described rhythmic discharges from the single motoneurones of extensor muscles during repetitive stimulation of a contralateral afferent nerve When the stimulation is not too infrequent, e g , when more than 10 a second, the rhythm of this reflex discharge bears no direct relation to the rhythm of the stimulation, but within limits it varies with the intensity of the reflex stimulus, which in turn is dependent on both the rate and the strength of the stimulation Somewhere in the reflex centre the reflex stimulus gives rise to the rhythmic discharge characteristic of the reflex response This paper describes an investigation into conditions underlying the production of such a rhythmic discharge from a motoneurone A preliminary account of some of the experimental work has already been published (Eccles and Hoff, 1931)

II Method

The animal (cat) having been anæsthetised, the 5th to the 9th post-thoracic dorsal roots were divided extradurally on the right side Decerebration by the trephine method followed immediately and the anæsthetic was discontinued The right soleus muscle was then completely freed from the surrounding tissues, care being taken to preserve its nerve and blood supply All the other nerves of both hind limbs have been divided peripherally In almost all experiments (see p. 490) a skin incision has been made completely circumscribing the right thigh. Shielded electrodes, E, have been applied to the central stump of the left popliteal or peroneal nerve, and to the intact right popliteal nerve, A, so that this can be stimulated in continuity, fig 1 The nerves have been prepared with their blood supply intact, and have always remained in good condition throughout the whole experiment.

Tetanic reflex responses of soleus muscle have been elicited by repetitive stimulation through electrodes E of the contralateral popliteal or peroneal nerve by condenser discharges from a neon tube apparatus By means of silver pins in the muscle the action-currents have been led to a string galvanometer, and they have been photographed by a falling plate camera at a plate speed of about 150 mm a second The nerve supply to soleus has then been cut down until there remains only one reflexly active motor unit This is indicated by the succession of identical action currents having a fairly regular rhythm, usually at a rate of 10 to 20 a second The cutting down has been done either peripherally as the nerve branches in the muscle or centrally by dividing the ventral root filaments Antidromic impulses to soleus motoneurones have been set up by stimulation of the intact right popliteal nerve by electrodes A (fig 1) with induction shocks rather stronger than maximal for the motor nerve fibres A Lucas pendulum has been employed to set up two or three of such induction shocks at known intervals apart

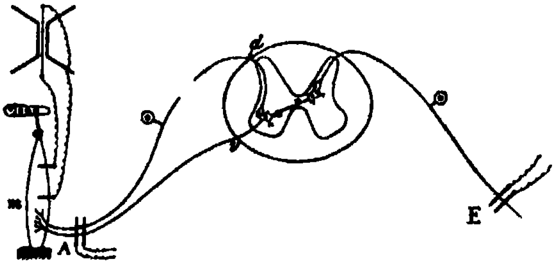

FIG 1 —Diagram of the nerve pathways and connections E shows electrodes on contralateral popliteal or peroneal nerve, and A electrodes on intact ipselateral popliteal nerve—the motor nerve to the soleus muscle *m* The afferent fibres of the ipselateral popliteal nerve have been cut as shown

The description of experimental technique previously given (Eccles and Sherrington, 1931, *a*) may be consulted for further details of the apparatus and the method of experiment Random variations have made it necessary to take a large number of observations in any one experiment, and altogether over 10,000 observations have been measured and calculated After each observation the contralateral stimulation has been stopped for a few seconds, and as a consequence the motoneurone has usually had a brief period of quiescence Under such conditions fatigue has barely been appreciable when a motoneurone has set up as many as 200,000 impulses in an experiment of 6 hours' duration

No difficulty has been encountered in deciding whether or not a series of action currents are due to the rhythmic discharge of a single motoneurone. As shown in observation 1, fig 2, Plate 24, the discharge from a single motoneurone is characterised by a rhythmic sequence of action currents identical in shape and size In observations 2 and 3 it is obvious that two motoneurones are discharging rhythmically

III Results

1 *General Features of the Crossed Extensor Reflex*

The features of the responses of a soleus motoneurone to a repetitive series of centripetal volleys in a contralateral nerve have already been described by Adrian and Bronk, and by Denny-Brown After a latent period of variable duration there are usually two discharges separated by an interval of about 10 σ (double responses) and then a pause followed by a fairly regular rhythmic discharge, at first slow, but gradually accelerating to a rate which is maintained for some seconds before a gradual deceleration sets in Immediately after cessation of the contralateral stimulation, the rhythmic discharge rapidly slows and usually soon stops In experiments with a well developed decerebrate rigidity, however, the rhythmic discharge may continue for an indefinite period

On occasion there may be two or more of the double responses at the start of the reflex, and when the rhythm is very slow it may be interrupted by double responses (Denny-Brown, 1929, fig 19) These double responses are always followed by pauses considerably longer than the normal response intervals (*cf* p 490) In one experiment a strong repetitive stimulation of a contralateral nerve frequently elicited an initial discharge of more than two impulses in quick succession, *e g*, close examination of the large initial action current in fig 3, Plate 24, reveals that it is made up of three superimposed action currents

2 *Investigations on the Rhythm of Reflex Discharge*

It may be assumed that a rhythmic reflex discharge is set up by a corresponding rhythmic activity central to the axon hillock of a motoneurone. An investigation of rhythmic reflex discharge therefore includes (1) a study of events during the cycle of rhythmic activity of the motoneurone , (2) a determination of the relation of the events upstream from a motoneurone to its rhythmic activity, *e.g.*, the incidence of excitatory or inhibitory impulses from neurones next upstream ; (3) an investigation into the setting up of rhythmic reflex impulses by the rhythmic activity of a motoneurone. In the present research only (1) has been directly studied, but some evidence bears on (2) and (3) as well

Rhythmic activity may be investigated by subjecting it to some influence which modifies the rhythm, e g , with the heart one may determine the effect on the rhythm produced by an extra-systole set up by direct stimulation of the pace-maker (Eccles and Hoff, 1932) Direct stimulation of a motoneurone is not possible with present technique, but a motoneurone may be acted on indirectly by taking advantage of the antidromic propagation of impulses in nerve fibres (Denny-Brown, 1929 , Eccles, 1931 , Eccles and Sherrington, 1931, *b*).

A. *The Effect of a Single Antidromic Impulse*

A single maximal stimulus, *e g* , a break-shock, applied to an intact motor nerve sets up in each nerve fibre two impulses travelling in opposite directions, one centrifugally evoking a motor twitch, the other antidromically to the motoneurone attached to that motor unit Such an antidromic impulse may be "backfired" into a motoneurone at any time during the cycle of its rhythmic reflex activity In each of the observations shown in figs. 4 and 5, Plate 24, an antidromic impulse has been backfired into a rhythmically discharging soleus motoneurone at various phases of its cycle The rhythm is interrupted by the antidromic impulse, and after a cycle longer than normal the rhythmic discharge recommences at the same rate as before It also appears that the more a rhythmic cycle is curtailed by the action of an antidromic impulse, *i e* , the shorter the ***curtailed cycle***, the longer is the immediately ***subsequent cycle***

In order to obtain more precise information concerning the relation, if any, between the durations of the ***curtailed cycles*** and the corresponding ***subsequent cycles***, a large number of observations have been made in each experiment. In all these observations the normal rhythmic activity is resumed after the cycle subsequent to the antidromic impulse. This rhythm, however, differs from the initial rhythm in phase, for the lengthening of the subsequent cycle does not fully compensate for the shortening of the curtailed cycle Attention need therefore only be focussed on the subsequent cycle and its relation to the curtailed cycle

Measurements from the action-currents of the muscle, *e.g* , between D, G and J in fig 6, give the time relations of events at the point where the antidromic impulse is set up, *i.e* , between K, E and L, for from there peripherally all the impulses concerned are travelling in the same direction in the same nerve fibre * But from the motoneurone to this point the antidromic impulse, EF, travels in the direction opposite to the reflex impulses, CK and HL, and so an allowance of twice the conduction time† has to be made in order to arrive at the correct time relations for the motoneurone, *i e*, between C, F and H

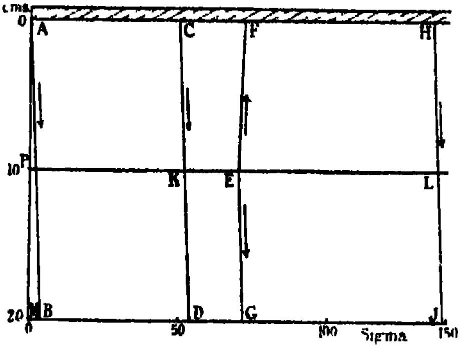

FIG 6—Diagram showing the passage of impulses to and from the spinal cord along a motor nerve fibre of soleus muscle Ordinates distance in centimetres from spinal cord A to muscle M, abscissæ time in sigma (*cf* Eccles and Sherrington, 1931, *a*, p 520). AB and CD are successive impulses discharged from the rhythmically active soleus motoneurone. At a point on the motor nerve a stimulus is applied setting up an antidromic impulse EF and an impulse EG passing to the muscle HLJ is the next impulse discharged from the motoneurone

The curtailed cycle, DG, has to be increased by this amount and the subsequent cycle, GJ, decreased

A series of such calculations confirms the original conjecture that the shorter the curtailed cycle the longer is the subsequent cycle, but there is considerable scatter in any series of observations That this is occasioned partly by variations in the frequency of the rhythmic discharge seems evident from the diminished scatter of any group of observations having similar frequencies of normal rhythmic discharge Classification of a large number of observations into a series of such frequency groups reveals that within limits (see p 490) comparable results are obtained with the different groups if the curtailed and subsequent cycles are expressed as fractions of the durations of the respective rhythmic cycles. Instead of working with such frequency grouping it has been found more convenient to reduce the cycles of each observation to fractions of the associated rhythmic cycle, and, in order to minimise the effects of irregularities in the rhythm, the duration of the rhythmic cycle has been taken as the average of the ten normal cycles centering about the antidromic impulse

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* Slowing of an impulse due to propagation in the relatively refractory period of a previous impulse would be infrequent and insignificant.

† This has been measured by a method identical with that described by Eccles and Sherrington (1931, *a*).

After calculation in this way the results of a series of observations have been plotted in fig 7 with the curtailed cycles as abscissæ and the corresponding subsequent cycles as ordinates Scatter is still considerable, but at any particular region the standard deviation is comparable with the standard deviation obtaining for the normal rhythm. The curve is slightly concave upwards and passes through the points 0·1 1·5, 1·0 · 1 0 Therefore when the motoneurone is about to discharge an impulse, an antidromic impulse backfired into it does not delay the discharge of the *next* impulse , but, when the

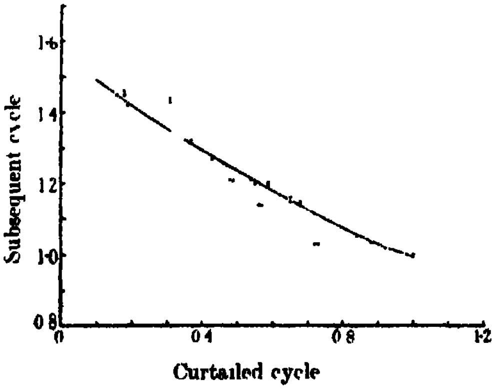

Fig 7 —Action of a single antidromic impulse on the rhythmic discharge of a soleus motoneurone Curtailed cycles plotted as abscissæ against subsequent cycles as ordinates, both being expressed as fractions of the average normal cycle

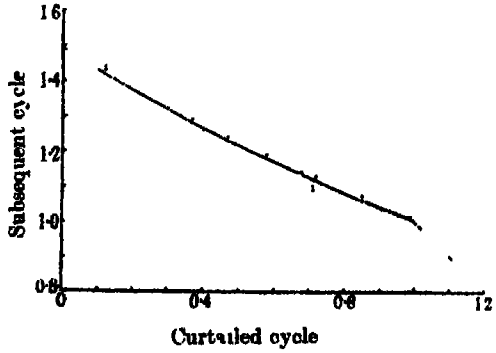

Fig 8 —As in fig 7 in another experiment

antidromic impulse curtails the rhythmic cycle after only 0·1 of it has elapsed, the subsequent cycle is 1·5 times the normal cycle.

It is impossible to "backfire" into a motoneurone earlier than about 0·1 of the cycle after a previous discharge, because an effective antidromic impulse cannot be set up until the immediately preceding reflex impulse has passed the stimulation point, *i.e.*, in fig. 6 K must precede E. An earlier antidromic impulse would be blocked by this outcoming reflex impulse. As the antidromic impulses were set up in a purely random relation to the rhythmic discharges, this would occur in a considerable number of observations. The action-currents of the muscle give no indication of this blocking, but it must have occurred with most of those observations having abscissal values greater than 1·0, and, owing to irregularities in the rhythm, with some also having slightly smaller values. Blocking probably occurs in about 6 to 10 per cent. of most series of observations. In such cases it would be anticipated that the rhythm would continue unchanged by the antidromic impulse, *i.e.*, the intervals before and after the antidromic impulse should sum to equal the duration of two normal cycles. Beyond 1·0 the points should therefore approximate to a line drawn at 45° as shown by the broken line in fig. 7. In this figure observations beyond 1·0 are not sufficiently numerous to give any significant curve, but at least they do not disagree with this expected curve.

In 12 of the 16 experiments similar series of results have been obtained, *e.g.*, fig. 8 from one of these experiments gives a curve resembling fig. 7. A curtailed cycle 0·1 times a normal cycle has in these experiments been followed by a subsequent cycle ranging from 1·43 to 1·65 of a normal cycle, and, when the antidromic impulse has acted on the motoneurone just as a reflex discharge was being set up, the subsequent cycle has been 0·98 to 1·07 times a normal cycle. Usually the curves have had a slight concavity upwards similar to those of figs. 7 and 8, but in one experiment the curve has been practically a straight line, and in one experiment the upward concavity was much greater.

The other four experiments have been characterised by a curve abnormally high throughout its whole course, even so high as to pass through the points 0·1 : 1·7 and 1·0 : 1·2, *i.e.*, the subsequent cycles have been abnormally long, fig. 9. Even when the antidromic impulse has been blocked by collision with the outcoming reflex discharge, the subsequent cycle has been abnormally long. This blocking must occur with a considerable number of observations having abscissal values greater or slightly less than 1·0, and yet in fig. 9, as shown by the ordinates, the subsequent cycle is always longer than normal. The other three experiments are similar in this respect. The abnormally long subsequent cycles in these four experiments are not, therefore, due to some abnormal action of the antidromic impulse. Some extraneous effect must be grafted on to the normal action of the antidromic impulse

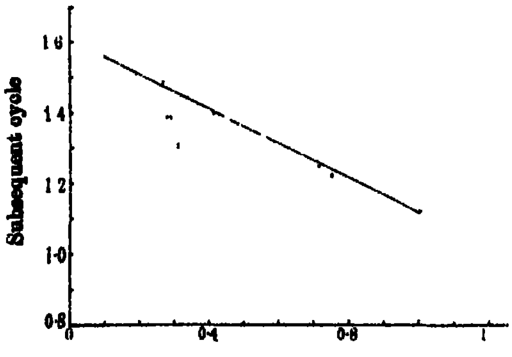

FIG. 9.—As in fig 7 in an experiment showing abnormally long subsequent cycles

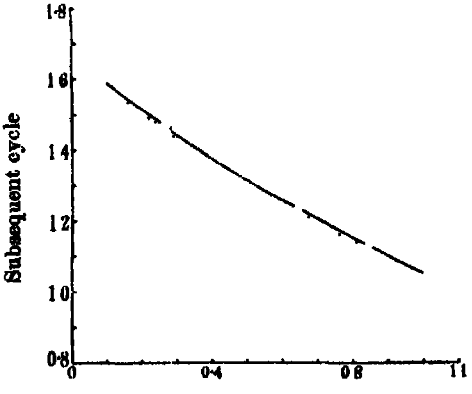

FIG 10—As in fig 7 in another experiment showing subsequent cycles somewhat longer than normal

In the first of these experiments the lengthening of the cycle was found to be produced by inhibitory impulses* set up from the skin by traction resulting from the muscular response to the antidromic stimulus, so in subsequent experiments special precautions were taken to denervate the skin (p. 483) In the other three experiments the abnormally long intervals may have been due to inhibitory impulses set up *directly* by the antidromic stimulus Since the segmental dorsal roots represented in the popliteal nerve, the 6th, 7th, 8th and 9th post-thoracic, were always completely severed, such impulses could have reached the spinal cord only by traversing afferent nerve fibres which entered the spinal cord by dorsal roots after passing headward in the sympathetic trunk for several segments (Kuntz and Farnsworth, 1928, Foerster, Altenburger and Kroll, 1929) It is probable that some of the 12 experiments provisionally classed as normal show the effect of this abnormal lengthening, *e g*, fig. 10, but to an extent less marked than in the above four experiments.

In six of the normal experiments an attempt has been made to determine if the curve varies according to the rate of the rhythmic discharge. Faster or slower rhythms were obtained by appropriate alterations in the strength of the repetitive stimuli eliciting the reflex, and each series of observations was plotted separately Fig. 11 is typical of three experiments in which the curve for the faster rhythms was higher than that for the slower In two other experiments alteration of the rhythm produced no significant effect, and in one the curve for the faster rhythms was lower than that for the slower

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* These impulses possibly travel along the cluneal nerves.

B *Grouped Discharges from Motoneurones*

Usually a regular rhythm at the original frequency is resumed after the subsequent cycle, but in 10 of our experiments the subsequent cycle has been at times followed by a double response and this in turn by a cycle longer than the normal rhythmic cycle, fig 12, Plate 24 Usually the regular rhythm is then resumed, but occasionally there have been two or more series of double responses followed by abnormally long cycles, fig 13, Plate 24 In such cases the intervals of the double responses progressively lengthen Double responses may also be set up by conditions other than those arising from the action of antidromic impulses (see p 486) The abnormally long cycle which always follows a double response indicates that the double response is due to a double discharge from a single motoneurone and not to the interfering discharges from two motoneurones This conclusion is confirmed by the invariable similarity of the two action currents in all cases except where the first interferes with a closely following second. The second may follow so closely that it is recognisable only by the long cycle following a slightly abnormal action current, fig 14, Plate 24, observations 1 and 3 Those cycles immediately subsequent to an antidromic impulse which are terminated by double discharges present no abnormalities when compared with corresponding

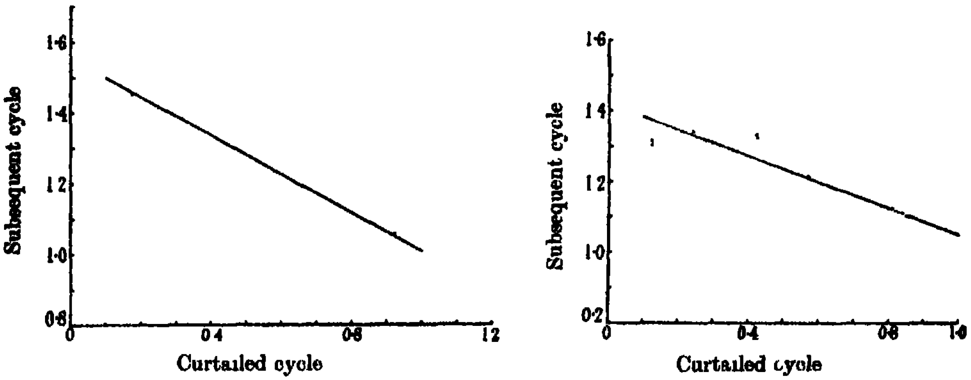

Fig. 11—As in fig 7 in another experiment The normal cycle had an average value of 0 065 seconds in fig 11A, and 0 094 seconds in fig 11B cycles terminated by the resumption of the normal rhythmic discharge. *Thus in double discharges the rhythmic cycle which would follow the normally occurring first discharge is brought prematurely to an end by the second discharge, and as a consequence this premature discharge is followed by a cycle longer than normal* These cycles may be termed respectively the *antecedent cycle* and the *subsequent cycle* of the *premature discharge.*

In most experiments the antecedent cycles of the premature discharge present all transitions between a duration as short as 0·1 of the normal cycle and the duration of the normal cycle itself The observations of fig 12, Plate 24, indicate that the shorter the antecedent cycle, the longer is the subsequent cycle A more precise examination of this relationship is afforded by fig 15 in which measurements from many observations in this experiment are calculated and plotted as ratios of the corresponding normal cycles in a way comparable with that adopted in figs 7 and 8 * The points fall about a curve very similar to that for the antidromic impulse, figs. 7 and 8. In fig. 16, a more extensive series from another experiment, the curve is more concave than in fig 15

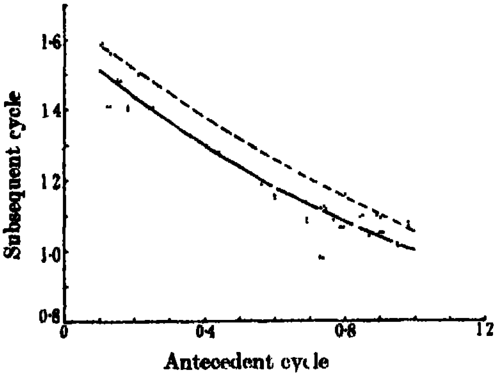

Fig 15

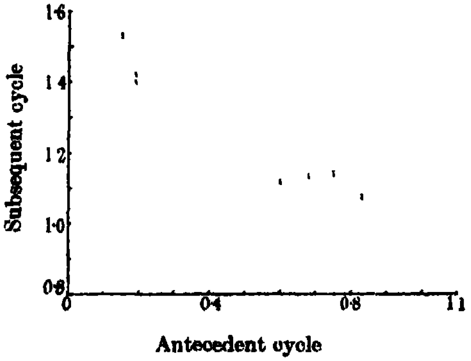

Fig 16

Fig 15 —Action of a premature discharge on the rhythmic cycle of a soleus motoneurone Antecedent cycles plotted as abscissæ against subsequent cycles as ordinates, both being expressed as fractions of the average normal cycle The broken line shows the curve for the single antidromic impulse in this experiment (fig 10) Fig 16 —As in fig 15 in another experiment. On account of their number only some of the observations having abscissal values less than 0·2 or greater than 0·7 have been plotted.

Curves from the whole series of experiments show that an antecedent cycle having a duration 0·1 times a normal cycle is followed by a subsequent cycle

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* No allowance is made for conduction time, as measurements from the action currents of the muscle give directly the time relations of the discharges from the motoneurone.

1·46 to 1·60 times a normal cycle. At the other extreme the curves always pass through the point 1·0 : 1·0 showing that an antecedent cycle of normal duration is followed by a subsequent cycle of similar duration; for, the second discharge not being then premature, there is of course a resumption of the regular rhythmic discharge immediately after the subsequent cycle of the antidromic impulse.

The curve for the premature discharges has always been closely similar to the curve for the antidromic impulses except in those cases where the latter was abnormally high. Then the curve for the premature discharges ran parallel to it passing as usual through the point 1·0 : 1·0, *e.g.*, in fig. 15 the broken line shows the curve for the antidromic impulses in that experiment (see fig. 10). Such a result is in accordance with the above-mentioned inference (p. 489) that the abnormally long subsequent cycles are due to some factor other than the antidromic impulse. Such a factor would not be operative with the double responses.

It may be concluded that with premature discharges the relation which antecedent cycles bear to subsequent cycles is the same as that obtaining between curtailed cycles and cycles subsequent to antidromic impulses. Thus, when similarly placed in the rhythmic cycle, the premature discharge and the antidromic impulse have similar effects on the rhythmic activity of a motoneurone. In other words an impulse travelling up the axis cylinder to a rhythmically active motoneurone affects the rhythm in the same way as a reflex discharge set up by that motoneurone (see also pp. 495, 498).

On general grounds (Eccles and Sherrington, 1931) it has been argued that an antidromic impulse should have an effect on a motoneurone similar to a reflex discharge, for both would traverse the same structures (probably the surface of the motoneurone including the dendrites), and, as the effects produced by an impulse in a peripheral nerve fibre do not depend on the direction of propagation, it was thought unlikely that any difference would exist with the motoneurone. This argument enhances the probability of the inference which may be drawn from the experimental evidence at present available. All this evidence shows similar actions on a motoneurone by an antidromic impulse and by a reflex discharge. The inference is that their actions are identical.

C. *The Effect of Two Antidromic Impulses.*

(i) *The Duration of the subsequent Cycle.*—It appears likely from the foregoing experimental observations that the lengthening of the cycle subsequent to the curtailment of a normal rhythmic cycle is an essential feature of the rhythmic activity of motoneurones It is of importance, therefore, to investigate the condition of the motoneurone during this subsequent cycle At present this can only be done by one form of experiment—the " backfiring " of a second antidromic impulse This second antidromic impulse may be set up after the first at any interval greater than the absolutely refractory period of the nerve fibre.

Fig 17, Plate 25, shows a series of observations with two antidromic impulses 8 σ apart, and in fig 18 from the same experiment this interval has been increased to 32 σ, and in fig 19 to 58 σ A comparison of these three figures suggests that the subsequent cycle is the longer, the shorter the interval between the two antidromic impulses The cycle curtailed by the first antidromic and the cycle subsequent to the second antidromic have been measured in each observation of a series, and after allowance for conduction time (*cf.* p 487) have been calculated as fractions of the corresponding normal cycles (averaged as on p 487) In fig 20 are plotted the results thus obtained for the series of observations with an interval of 8 σ between the two antidromic impulses, while in fig 21 the interval was 32 σ, and in fig 22 it was 58 σ

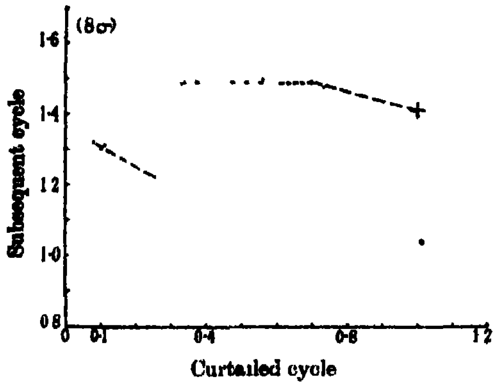


FIG 20

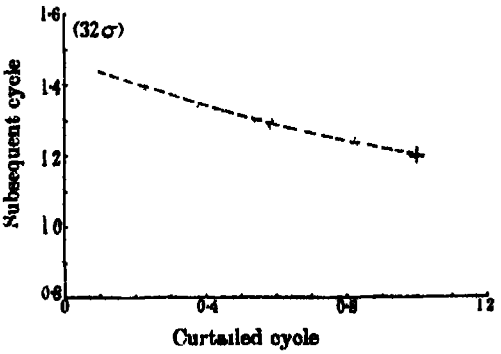


FIG 21

FIG. 20—Action of two antidromic impulses on the rhythmic discharge of a soleus motoneurone Curtailed cycles plotted as abscissæ against cycles subsequent to the second antidromic as ordinates, both being expressed as fractions of the average normal cycle. The lines are theoretical curves (see p 495) Interval between the antidromic impulses is 8 σ. FIG 21—As in fig 20 and in the same experiment, but the antidromic interval is increased to 32 σ

With the exception of the series of low points for the greatly curtailed cycles in fig. 20 (to be considered on p. 498) the observations with the interval of 8 σ tend to lie about an almost horizontal line, the subsequent cycle being therefore almost unaffected by the length of the curtailed cycle On the other hand, when the interval was 32 σ, the subsequent cycles are the longer the shorter the curtailed cycles, but they tend to be always shorter than when the interval was 8 σ With intervals of 58 σ the subsequent cycles are still shorter Similar series of curves have been obtained in many of the 14 experiments in which

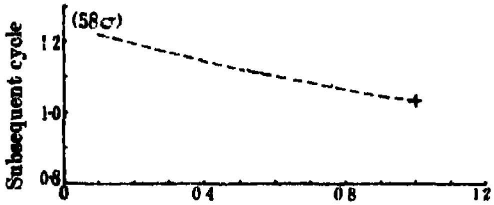


Fig 22 —As in fig 21, but the antidromic interval is now 58 σ

double antidromic impulses have been set up, but in four the condition causing the low values to the left of fig 20 has been much more prominent (*cf* p 498), and in four other experiments this effect has been present only with intervals shorter than 8 σ, fig 23 shows observations from one of these experiments

(ii) *Derivation of the Curve for Two Antidromic Impulses* —There now arises for consideration the possibility of determining a relationship between the curves for single and double antidromics which allows the derivation of the latter from the former Consider the condition where the curtailed cycle has a duration of almost 1 0, *i e*, the first antidromic impulse acts on the motoneurone just as a reflex discharge is being set up Since we have seen reason to consider the action of the antidromic impulse as identical with that of a reflex discharge, the second antidromic impulse would be expected in the above case to act on the motoneurone exactly as if it had been preceded at the same interval by a reflex discharge instead of the first antidromic The subsequent cycle should then be calculable from the single antidromic curve Points calculated in this way are indicated by the crosses at abscissal values of 1 0 in figs 20, 21, 22, 23, 27 and 28, and are in satisfactory agreement with the experimental results With the exception of a few cases where antidromic impulses were set up at very short intervals apart (*cf* p 498), the agreement has also been satisfactory in all other experiments The conclusion that antidromic impulses and reflex discharges affect a motoneurone similarly therefore receives additional support

It is impossible to calculate any other points on the curve for the double antidromics without making an assumption It seems simplest to assume as a first approximation that throughout the *latter* part of the cycle subsequent

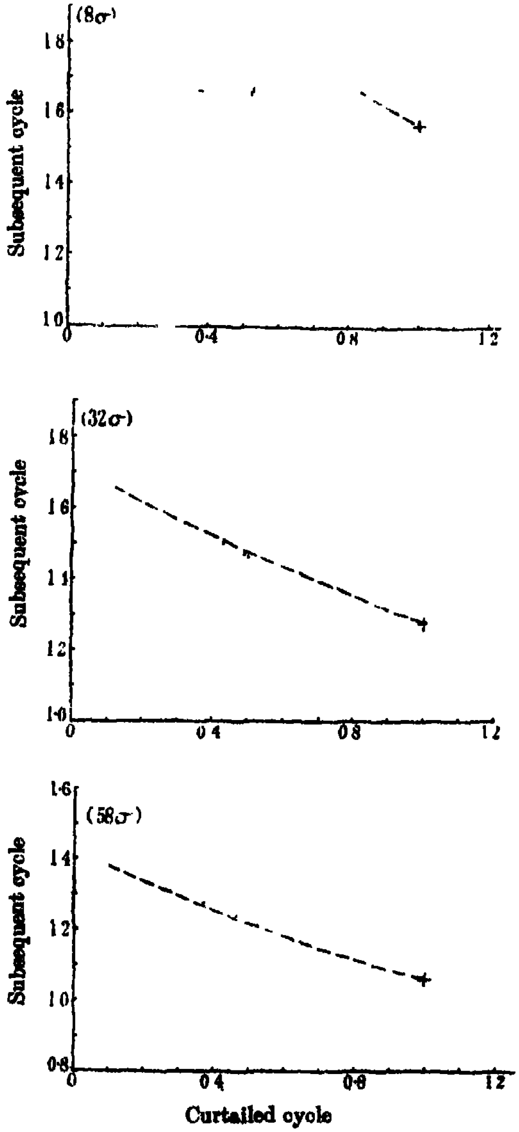
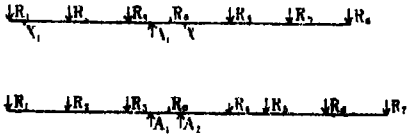


Fig 23 —As in figs 20, 21 and 22, but in another experiment

to a single antidromic impulse the motoneurone is in a condition identical with that obtaining in a normal cycle *at similar intervals before the next reflex discharge*. For example, in fig. 24, R_1, R_2, R_3, R_4, R_5, R_6 represent the successive discharges of a motoneurone whose rhythmic cycle is interrupted by the antidromic impulse A_1 with its subsequent cycle A_1R_4. If R_0 is placed so that R_0R_4 equals R_1R_2, R_2R_3, etc., then it is assumed that the condition of the motoneurone between R_0 and R_4 is identical with its condition at corresponding points in the normal cycles R_1R_2, etc., *e.g.*, at the points X_0 and X_1 if $R_0X_0 = R_1X_1$.

By adopting this assumption it is possible to calculate the interval subsequent to the second of two antidromic impulses if given the curve with a single antidromic impulse. In fig. 25, A_1 and A_2 represent the two antidromic



FIGS. 24 AND 25.

impulses interrupting a rhythmic discharge $R_1R_2R_3$. If A_1 alone acted on the motoneurone the next discharge would occur at a point R_4 after a subsequent cycle A_1R_4 which is derivable directly from the single antidromic curve for any given duration of the curtailed cycle R_3A_1. By assumption the motoneurone at A_2 is in a condition identical with that obtaining at an interval R_0A_2* after a reflex discharge ($R_0R_4 = R_1R_2$, etc.). The cycle A_2R_5 subsequent to A_2 should therefore have a duration identical with the cycle subsequent to a single antidromic impulse which acts after a curtailed cycle equal to R_0A_2. In this way it is possible to calculate cycles subsequent to A_2 corresponding to chosen durations of the cycles curtailed by A_1, and curves expressing this relationship have been drawn in figs. 20, 21, 22, 23 and 28 as broken lines.

In these and other experiments such curves appear to agree with the experimental points sufficiently well to justify the initial assumption as a first approximation. It will be noticed that only a portion of the total curve is derivable when there are short intervals between the antidromic impulses. If in fig. 25 A_1A_2 is so short that A_2 lies between A_1 and R_0, it is impossible to calculate the position of R_5. Even when A_2 lies just after R_0, the position of R_5 can only be calculated by using an extrapolation of the single antidromic curve, *e.g.*, that shown by the broken lines in figs. 7 and 8.

A study of the experimentally obtained points over that part of the double antidromic curve not derivable as above reveals that the curve is almost horizontal As the curtailed cycle is made shorter and shorter, a stage is reached where further abbreviation produces no accompanying lengthening of the subsequent cycle, and probably therefore no additional change in the reflex activity of the motoneurone Thus a basal condition seems to be produced if A_2 coincides with R_0 If A_2 is to the left of R_0 there is no additional effect on the rhythmic activity of the motoneurone This basal condition would therefore be produced by an effect equivalent to that which would be produced if an antidromic impulse acted at the same time as a reflex discharge,* or if two antidromic impulses acted simultaneously * This inference allows the completion of the missing parts of the theoretical curves by the horizontal dotted lines in figs 20 and 23 †

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* R_0A_2 is calculated as $A_1A_2 - (A_1R_4 - R_0R_4)$.

(iii) *The Refractory Period of the Motoneurone*—In fig 20 all points from observations with curtailed cycles less than 0 26 of the normal cycle fell in a group distinct from the other observations Since the broken line drawn through these observations is the curve which would be given if the first antidromic impulse alone acted on the motoneurone,‡ it is apparent that the second antidromic had no effect on the rhythmic activity of the motoneurone

In three experiments the second antidromic at an interval of 8 σ after the first was ineffective even when the first acted at the end of a normal cycle, fig 26, *i e*, when the curtailed cycle was almost equal to a normal cycle With four exceptions all the observations of fig 26 having abscissal values greater than 1 0 have also such short subsequent cycles that the second antidromic could have had little if any effect on the reflex activity of the motoneurone But in most of these observations the first antidromic impulse would be blocked by collision with the outcoming reflex impulse (*cf.* p 489) It therefore seems that the ineffectiveness of the second antidromic impulse may arise when it is preceded at a short interval by either a reflex discharge or an antidromic impulse The reflex impulse, however, always precedes the second antidromic by an interval longer than the antidromic interval (otherwise it would not collide with the first antidromic), and in some cases this lengthening might be as great as twice the conduction time plus the duration of the absolutely refractory period at the point where the antidromic stimulus was applied This would account for the effectiveness of the second antidromic in the four exceptional observations of fig 26 without the necessity of postulating any difference between the actions of reflex discharges and antidromic impulses (*cf* p 493) Similarity of the refractory periods following reflex discharges and antidromic impulses is also indicated by the other two experiments in which the second antidromic was ineffective with all durations of the curtailed cycle

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* Refractory period of course makes this simultaneous action impossible.

† *Note added in proof, March 24*, 1932—If two antidromic impulses depress the rhythmic activity of a motoneurone to a basal condition, no further depression should be produced by the action of three or more of such impulses This is confirmed by experiment, for the cycle subsequent to three or more antidromic impulses is no longer than that obtaining with a basal depression by two antidromic impulses It may, however, be shorter owing to blocking of the last antidromic impulse or impulses by an absolutely refractory state set up by the previous impulse (p 502)

‡ This line is drawn lower than the curve, fig 8, for this experiment, because the subsequent cycles in fig 20 were measured from the second antidromic impulse.

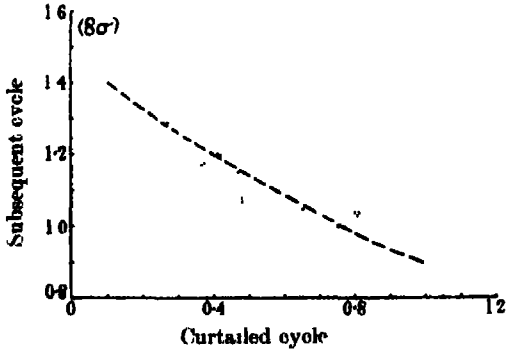

Fig 26 —As in fig. 20 but in another experiment The broken line shows the expected curve if the first antidromic alone was effective

In all experiments both antidromic impulses affect the rhythmic cycle if the interval between them is sufficiently long Thus a series of observations at antidromic intervals of 32 σ, fig 29, Plate 25, is shown in fig 27 (from the same experiment as fig 26) When the curtailed cycle is less

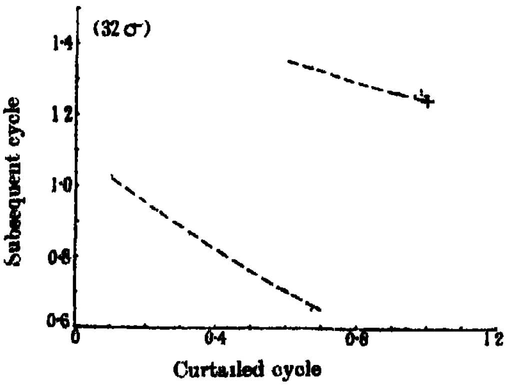

Fig 27.—Same experiment as fig. 26, but the antidromic interval is increased to 32 σ

than 0·6 of a normal cycle, the observations fall into a low group (*cf* observations 2 and 4, fig. 29), when greater than 0·6, most observations fall into a high group which agrees well with the curve for the double antidromics

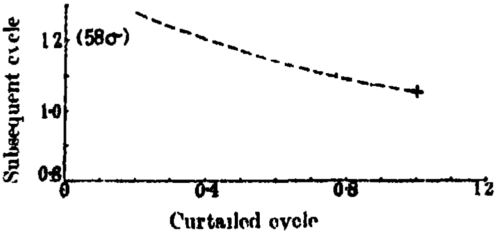

FIG. 28.—Same experiment as fig. 26, but the antidromic interval is increased to 58 σ

derived as above from the single antidromic curve. It therefore seems probable that the second antidromic has a normal action in the observations of this latter group. Even with observations having a curtailed cycle less than 0·6, the second antidromic appears to cause a small lengthening of the subsequent cycle, for the points lie about a curve rather higher than the curve as drawn in fig. 27 for the single antidromic (derived from fig. 7 by making allowance for the antidromic interval). Observations with curtailed cycles between 0·6 and 0·8 of a normal cycle tend to scatter widely, and some points deviate so far from either of the main groups that there seems to be a likelihood of different grades of partial effect being produced by the second antidromic impulse. Finally, in this experiment fig. 28 shows the series with an interval of 58 σ between the antidromics. Since the theoretical curve expresses the relationship so well, it may be inferred that at this interval the second antidromic impulse has a normal action for all durations of the curtailed cycle.

In some experiments where series of observations have been made with many different intervals between the two antidromic impulses, a break in the curve such as that shown in figs. 20 and 27 occurred with several of these intervals. Such series are shown in fig. 30, where there are definite breaks in those series with intervals of 8 σ, 12 σ, 16 σ and 20 σ between the antidromic impulses, and even with an interval of 25·6 σ the second antidromic has only a partial effect when the curtailed cycle is very short. At an interval of 32·2 σ, however, the second antidromic seems to have a full effect with all durations of the curtailed cycle. This break in the curve happens with longer curtailed cycles the shorter the intervals between the antidromic impulses. Thus in fig. 30 the break is at about 0·59 for 8 σ, 0·58 for 12 σ, 0·47 for 16 σ, 0·40 for 20 σ, and 0·22 for 25·6 σ. These values for the curtailed cycles are only approximate because the sharpness of the break is blurred by overlap. The same general tendency is observable in the three other experiments in which a break in the curve occurred with several different intervals between the antidromic impulses, *e.g.*, in one the break was at 0·55 for 5 *σ*, 0·41 for 8 *σ*, 0·34 for 16 *σ*, 0·24 for 24 *σ*, and 0·16 for 32 *σ*.

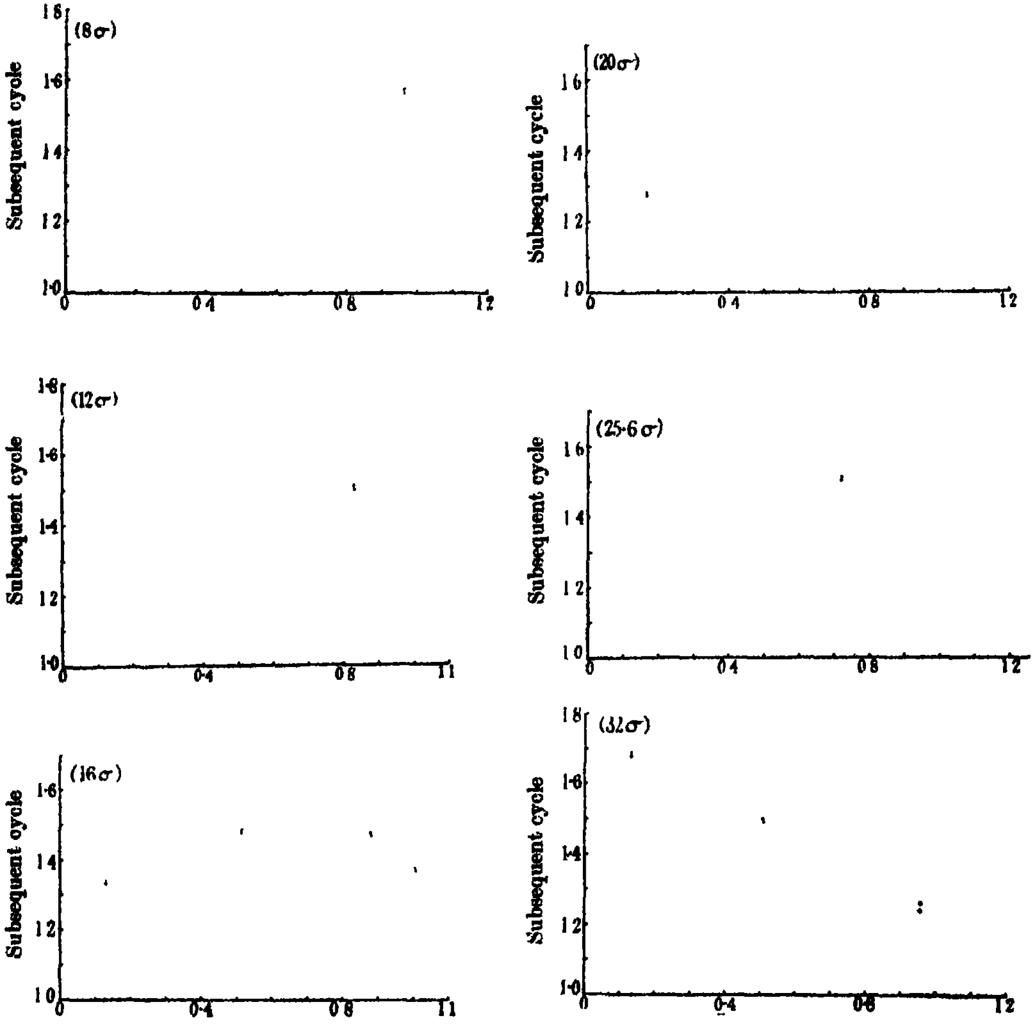

Fig. 30.—As in fig. 20, but in another experiment. Series are shown with antidromic intervals of 8 *σ*, 12 *σ*, 16 *σ*, 20 *σ*, 25·6 *σ*, and 32 *σ*.

There now arises the question of the explanation to be adopted for the ineffectiveness of the second antidromic impulse It cannot be that the rhythmic activity of the motoneurone is so depressed by the first antidromic that the second antidromic has no further effect, for (as shown by the longer subsequent cycles) this rhythmic activity is depressed much more when both the antidromics are acting effectively at the longer curtailed cycles, fig 27. The ineffectiveness of the second antidromic must therefore be due to its inability to reach the seat of the rhythmic activity Absolutely refractory state is the only known condition of nervous tissue which could account for this blocking, because it has been shown that inhibition is powerless to prevent an antidromic impulse from reaching the locus where reflex discharges are set up (Eccles and Sherrington, 1931, *c*) The second antidromic impulse is blocked by absolutely refractory tissue either before or at the seat of the rhythmic activity

The experimental results already described show that the duration of this absolutely refractory period varies greatly from one motoneurone to another, *e g.*, in one experiment it was never as long as 6 σ and in another it even exceeded 60 σ The experimental results also show that the length of the curtailed cycle exercises an important influence on the duration of the absolutely refractory period in those motoneurones where this duration is not always brief. Thus in one of the experiments quoted above, the absolutely refractory period was less than 5 σ when the curtailed cycle was longer than 0·55 times a normal cycle, but it was longer than 32 σ with curtailed cycles less than 0·16 of the normal cycle In other experiments there have been comparable variations, *e g*, from 6 σ to 25 6 σ and from 8 σ to 32 σ

If the absolutely refractory period following the first antidromic impulse is actually located at the rhythmically active part of a motoneurone, then it may be related to the lengthening of the cycle subsequent to this antidromic impulse Such an association would seem to be supported by those previous observations which show that both the absolutely refractory period and the subsequent cycle lengthen progressively as the curtailed cycle is shortened. Thus, during the initial phase of the subsequent cycle, the rhythmically active part would be absolutely refractory, and the longer this absolutely refractory period, the longer would be the subsequent cycle. The correspondence is shown in figs. 31 and 32, where the continuous line shows the relation between subsequent cycle and curtailed cycle, and the broken line the relation between absolutely refractory period and curtailed cycle, all values being expressed as fractions of the normal cycle In both these experiments the absolutely refractory period, shown by the shaded portion, appears to bear some relation to the duration of the subsequent cycle, but in other experiments with similar curves for the subsequent cycle the absolutely refractory period increases very little as the curtailed cycle is shortened, fig 33, and in one experiment it was uniformly short for all durations of the curtailed cycle, fig. 34 It seems, therefore, that the relation between absolutely refractory period and subsequent cycle apparently shown in figs 31 and 32 is accidental, and that the absolutely refractory period has no significant influence on the duration of the subsequent cycle

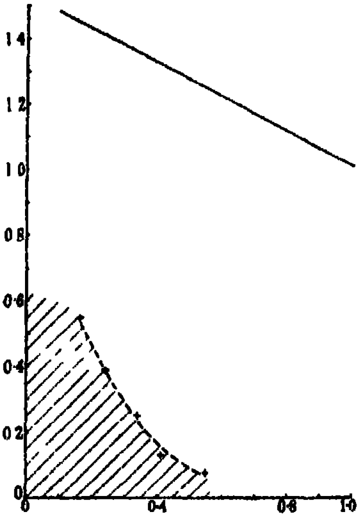

Fig 31

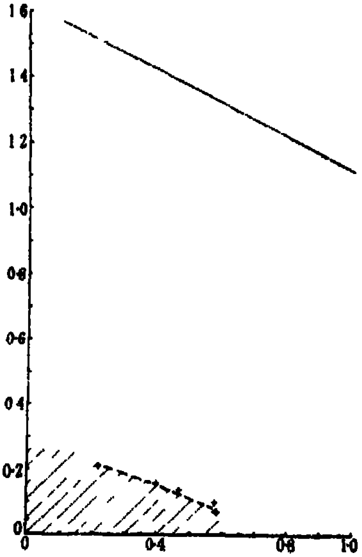

Fig 32

Fig. 31 —The continuous line shows the single antidromic curve and the broken line expresses the relation between the curtailed cycle and the subsequent absolutely refractory period The crosses show actually determined values Both curves are drawn on the same co-ordinates—subsequent cycles as ordinates, curtailed cycles as abscissæ, both being expressed as fractions of the average normal cycle The absolutely refractory part of the subsequent cycle is shaded in Fig 32 —As in fig 31 in another experiment

Might not an important influence, however, be exercised by the relatively refractory period which presumably lies between the conditions of absolute refractoriness and subsequent complete recovery? Experimental evidence shows that both in peripheral nerve and in the motoneurone their relatively refractory periods bear a similar relation to their respective absolutely refractory periods (Eccles, 1931), and so it seems improbable that there would be any wide independent variation under the conditions here considered. Figs. 33 and 34 show that such a wide variation would have to be postulated if an attempt were made to correlate the duration of the subsequent cycle with the length of the relatively refractory period.

Thus it seems that the duration of the subsequent cycle after an antidromic impulse is mainly determined by some factor or factors other than the length of the absolutely or relatively refractory period set up by it

In figs 20, 27 and 30 it will be noticed that there is no abnormal lengthening of subsequent cycles in which the second antidromic acted just after the end of the absolutely refractory period set up by the first antidromic These subsequent cycles have a duration corresponding closely to the duration calculated according to the assumption made on p. 497, and also to the durations found in those experiments where the refractory period was not lengthened, fig. 23 It would seem that if the refractory period following the second antidromic suffers any additional augmentation, this is not reflected in an additional lengthening of the subsequent cycle.

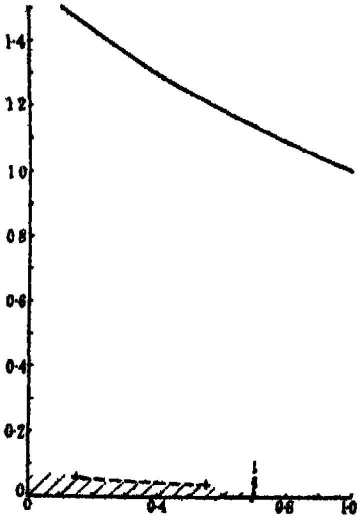

Fig. 33.—As in fig 31 in another experiment.

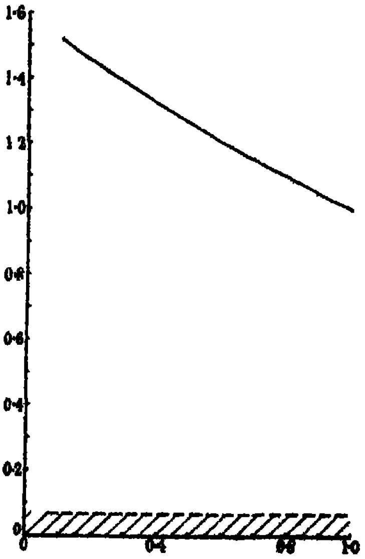

Fig 34 —As in fig 31 in another experiment.

IV Discussion.

Rhythmic discharges of impulses from a motoneurone must be occasioned by corresponding fluctuations in the "activity" of a rhythmic centre. A certain state of the rhythmic centre is necessary to set up a reflex discharge. The

FIG 2

FIG 3

FIG 5

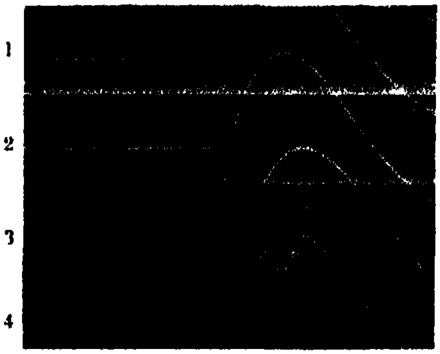

FIG 4

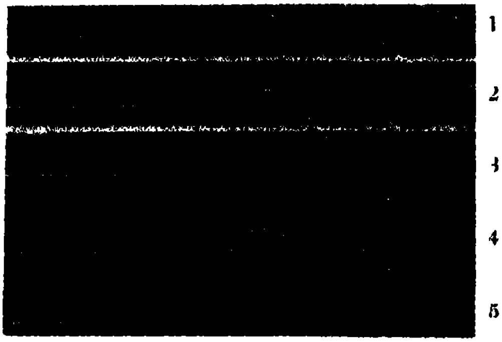

FIG 12

FIG 13

FIG 14

Fig. 17

Fig. 18

Fig. 19

Fig. 29

"activity" of the rhythmic centre is a measure of this state, *i e.*, it is a measure of the propensity of the rhythmic centre to set up such a discharge. Each discharge must be preceded by an increase in the "activity" of the rhythmic centre to a threshold intensity, and an immediate diminution in "activity" must result from the reflex discharge Such general considerations would apply to the setting up of any rhythmic condition of a similar character, *e.g*, the heart beat.

Since the rhythmic discharge of a soleus motoneurone is resumed with an altered phase after interruption by an antidromic impulse (p 485), the rhythmic centre must be acted on by this impulse It has elsewhere been argued that an antidromic impulse in a motor nerve fibre does not pass beyond the motoneurone with its dendrites (Eccles, 1931) Each motoneurone has, therefore, an intrinsic rhythmic centre The rhythm of its discharge is not impressed on it from without, for example, by internuncial neurones next upstream A rhythmically discharging motoneurone must be subjected to a continuous and more or less uniform bombardment by excitatory impulses from these internuncial neurones The stimulus of this bombardment activates the rhythmic centre of the motoneurone as evidenced by the discharge so set up. The more intense the bombardment, the faster is the rhythm of this discharge

The sequence of events during this rhythmic discharge has been investigated by systematic examination of the effects produced by antidromic impulses The effect produced by the action of an antidromic impulse on a motoneurone has a special interest since it is identical with that produced by a reflex discharge (p 493)

A single antidromic impulse interrupting the rhythmic cycle of a motoneurone is followed by a cycle longer than the normal rhythmic cycle, and this subsequent cycle is the longer the shorter the cycle curtailed by the antidromic impulse (p 485). It seems therefore that the "activity" of the rhythmic centre is depressed by an antidromic impulse more than by a normal reflex discharge, and this depression is the greater, the earlier the action of the antidromic impulse The antidromic impulse is able to do this, because, unlike the normal reflex discharge, it acts before the "activity" has reached threshold. Again the earlier it acts in the rhythmic cycle, the less intense will it find the "activity," which as a consequence will be depressed to a lower level, hence the longer subsequent cycle

A second antidromic impulse acting during the cycle subsequent to a previous antidromic is followed by a cycle having a duration corresponding closely to that calculated from the action of a single antidromic impulse if it be assumed that the rhythmic centre of a motoneurone is in the same condition of "activity" at similar intervals before the next reflex discharge (p. 497). The premature reflex discharge met with under certain conditions (p. 491) is exceptional in this respect The lengthened cycle following the premature discharge (p 493) indicates that the "activity" of the rhythmic centre has been depressed by it to the same level as by an antidromic impulse acting at a similar time The "activity" of the motoneurone should, therefore, be below the threshold intensity when the premature discharge is set up It is not known how the discharge is set up under such circumstances

The action of two or more antidromic impulses indicates that there is a basal level below which the "activity" cannot be depressed. This basal level of "activity" seems to be separated from the threshold by an amount equal to twice the depression produced by a single antidromic impulse or by a single reflex discharge

The refractory period following antidromic impulses or reflex discharges does not seem to exercise an important influence on the "activitiy" of the rhythmic centre (p 503)

The experimental evidence does not allow any further direct inferences concerning the processes in the rhythmic centre other than the above description of the changes in its "activity," but in the next section a more detailed account has been attempted by means of a mathematical treatment.

V. THEORETICAL TREATMENT OF REFLEX DISCHARGE.

The following assumptions have been made in order to allow a mathematical treatment of the rhythmic "activity" of a motoneurone

1 During the rhythmic responses of a motoneurone in a tetanic crossed extensor reflex the incident excitatory impulses are producing at a uniform rate a condition called the central excitatory state, c e.s. This assumption should be justifiable when random variations are minimised by treating statistically a large number of observations as has been done in the present research

2 This c.e.s. undergoes a spontaneous subsidence at a rate proportional to its intensity.

3. If the c.e.s. of a motoneurone attains a certain intensity called the threshold, a reflex discharge is set up. The threshold being assumed constant, the "activity" will be measured by the intensity of the c e.s.

4. Consequent on a reflex discharge there is an instantaneous inactivation of one-half the threshold intensity of c.e.s. An antidromic impulse has a

similar action (p. 492) provided that at least one-half of the threshold intensity of c.e.s is present at the time of its action—if less is present, it would be all inactivated. This fourth assumption is based on evidence from two sources. (*a*) The duration of the cycle subsequent to two antidromic impulses may be satisfactorily explained if it be assumed that a basal condition* of the motoneurone is reached by an effect equivalent to that produced if two antidromic impulses could act simultaneously on a motoneurone excited at threshold intensity, *i e*, each inactivates half the threshold intensity (*b*) Spontaneous reflex discharges from motoneurones frequently occur in groups of two, which, when very close together, depress the " activity " of the motoneurone almost to its basal level, as indicated by the length of the subsequent cycle, *i e*, each inactivates one-half of the threshold intensity.

The production of a rhythmic reflex discharge according to the conditions postulated is illustrated in fig. 35, where intensities of c.e s are plotted as

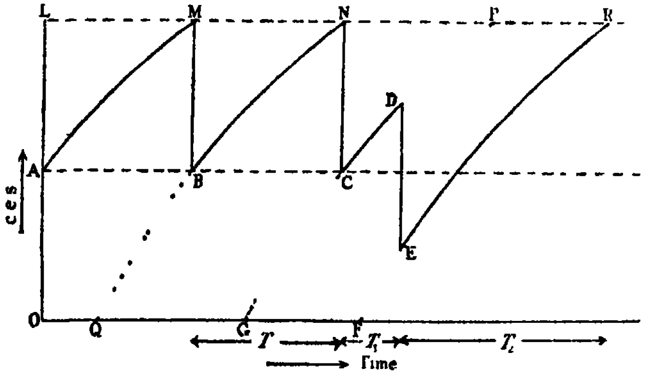

Fig. 35—Schematic representation of rhythmic discharge and the action of a single antidromic impulse. For description see text.

ordinates against time as abscissæ. OF is the line of zero intensity of c e s. According to the fourth assumption the intensity of c e s, OA, at the point A just after a reflex discharge, equals half the threshold intensity OL. As a result of its uniform production and spontaneous subsidence, c e s. is represented as increasing in intensity during normal rhythm along the line AM to again attain threshold at M, where the next reflex discharge is set up. The intensity of c.e.s. falls from M to B, and then increases along the line BN, till at N another reflex discharge is set up, with a consequent fall of c.e.s to C followed by an increase to P, and so on.

---

* In this condition no c.e.s. would be present.

NB is continued backwards to Q along the line which the c.e.s. would have taken if it had increased from zero intensity to N. Let p be the uniform rate of production of c.e.s., and s the rate of subsidence of unit intensity of c.e.s. Then if x is the intensity of c.e.s. present at a point on the line QBN at a time t seconds after Q,

$$\frac{dx}{dt} = p - sx$$

the general solution of this equation is

$$x = \frac{p - C\varepsilon^{-st}}{s},$$

where C is an arbitrary constant. When $t = 0$, $x = 0$, hence in the above equation $C = p$, therefore

$$x = \frac{p}{s}\left(1 - \varepsilon^{-st}\right) \tag{1}$$

and

$$t = -\frac{1}{s}\log_e\left(1 - \frac{sx}{p}\right) \tag{2}$$

Therefore the time to produce the threshold intensity, k, *i.e.*, the time between Q and N, equals

$$-\frac{1}{s}\log_e\left(1 - \frac{sk}{p}\right) \text{ seconds,} \tag{3}$$

and the time to produce an intensity $\frac{1}{2}k$, *i.e.*, the time between Q and B equals

$$-\frac{1}{s}\log_e\left(1 - \frac{sk}{2p}\right) \text{ seconds,} \tag{4}$$

therefore

$$T = -\frac{1}{s}\log_e\left(1 - \frac{sk}{p}\right) + \frac{1}{s}\log_e\left(1 - \frac{sk}{2p}\right) \text{ seconds,}$$

where T is the length of the normal cycle MN,

$$= \frac{1}{s}\log_e\left(\frac{2p - sk}{2p - 2sk}\right) \text{ seconds,} \tag{5}$$

and

$$k = \frac{2p\left(\varepsilon^{sT} - 1\right)}{s\left(2\varepsilon^{sT} - 1\right)}. \tag{6}$$

It is now possible to arrive at a relation between the curtailed and the subsequent cycle. By assumption an antidromic impulse acting at a point D, fig. 35, inactivates an intensity $\frac{1}{2}k$ of c.e.s., as shown by the line DE, and c.e.s. then increases along the line ER to regain a threshold intensity at R, where a reflex discharge is set up. If the time from C to D is T_1 seconds, and from D to R is T_2 seconds, it is required to find the relationship between T_1 and T_2.

PC is continued to G in the same way as NB is to Q. Since the time from G to C equals that from Q to B (equation (4)), the point D, T_1 seconds after C, will be

$$T_1 - \frac{1}{s}\log_e\left(1 - \frac{sk}{2p}\right) \text{ seconds after G,}$$

and therefore substituting in equation (1) the intensity of c.e.s. at D

$$= \frac{p}{s}\left[1 - \varepsilon^{-s\left\{T_1 - \frac{1}{s}\log_e\left(1 - \frac{sk}{2p}\right)\right\}}\right]$$

$$= \frac{p}{s}\left\{1 - \varepsilon^{\log_e\left(1 - \frac{sk}{2p}\right) - sT_1}\right\}$$

Substituting the value for k from equation (6), the intensity of c.e.s. at D

$$= \frac{p}{s}\left(1 - \varepsilon^{\log_e \frac{\varepsilon^{sT}}{2\varepsilon^{sT} - 1} - sT_1}\right),$$

$$= \frac{p}{s}\left\{1 - \frac{\varepsilon^{s(T-T_1)}}{2\varepsilon^{sT} - 1}\right\} \tag{7}$$

At the point E the c.e.s. will have an intensity ½k less than at D, and so substituting the value for k from equation (6), the c.e.s. at E

$$= \frac{p}{s}\left\{1 - \frac{\varepsilon^{s(T-T_1)}}{2\varepsilon^{sT} - 1}\right\} - \frac{p}{s}\left(\frac{\varepsilon^{sT} - 1}{2\varepsilon^{sT} - 1}\right),$$

$$= \frac{p}{s}\left\{\frac{\varepsilon^{sT} - \varepsilon^{s(T-T_1)}}{2\varepsilon^{sT} - 1}\right\} \tag{8}$$

RE is continued to F along the line which the c.e.s. would have taken if it had increased from zero intensity to E. T_2, the time between E and R, equals the time between F and R minus the time between F and E. The time from F to E is the time required to build up an intensity of c.e.s. equal to

$$\frac{p}{s}\left\{\frac{\varepsilon^{sT} - \varepsilon^{s(T-T_1)}}{2\varepsilon^{sT} - 1}\right\} \quad \text{(equation (8)),}$$

and therefore substituting in equation (2) the time from F to E

$$= -\frac{1}{s}\log_e\left\{1 - \frac{\varepsilon^{sT} - \varepsilon^{s(T-T_1)}}{2\varepsilon^{sT} - 1}\right\} \text{ seconds,}$$

therefore

$$T_2 = -\frac{1}{s}\log_e\left\{1 - \frac{2(\epsilon^{sT}-1)}{2\epsilon^{sT}-1}\right\} + \frac{1}{s}\log_e\left\{1 - \frac{\epsilon^{sT}-\epsilon^{s(T-T_1)}}{2\epsilon^{sT}-1}\right\} \text{ seconds,}$$

$$= \frac{1}{s}\log_e\{\epsilon^{s(T-T_1)} + \epsilon^{sT} - 1\} \text{ seconds} \qquad (9)$$

This mathematical treatment may be considered in relation to several aspects of the experimental observations.

(*a*) The curve expressing the relation between the curtailed and subsequent cycles of a single antidromic impulse, *i.e.*, between T_1 and T_2.

In equation (9) if $T_1 = T$, then $T_2 = T$, so curves from this equation also pass through the points 1·0, 1·0 when plotted on the same co-ordinates as in figs. 7 and 8. Again, if a value is substituted for the constant s in equation (9) so that this equation expresses the experimental relationship between T_1 and T_2 for one value of T_1, it is also found to express this relationship for all other values of T_1. Thus in fig. 8 a value $s = 13·4$ is arrived at by graphic solution of the equation obtained by substituting in equation (9) $T_1 = 0·1T$, $T_2 = 1·43T$, see fig. 8, and $T = 0·074$ seconds. The curve obtained when this value of s is substituted in equation (9) is shown in fig. 36. It agrees well with the experimental observations at all values of T_1. In the same way a value of s can be chosen so that equation (9) agrees closely with the experimental curve in all cases where this curve has the usual slight upward concavity shown in figs. 7 and 8 (*cf.* p. 489). Equation (9) cannot be applied to abnormally high curves, *e.g.*, fig. 9 unless allowance is made for the extra lengthening.

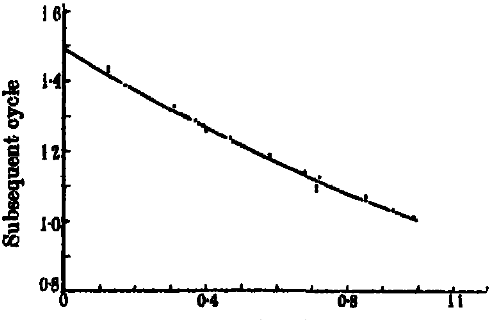

FIG. 36.—Observations plotted as in fig. 8, but the curve drawn is calculated from equation (9).

By assumption the value of s is independent of the rate of building up of c.e.s. on a motoneurone, and hence of the rate of its rhythmic discharge.

Equation (6), therefore, allows a determination of p in terms of k for any particular rate of reflex discharge Table I gives values of s for different motoneurones and also the values of p/k for different rates of discharge of these motoneurones From equation (5), T is infinite when $p/k = s$ Table I, column 6, shows that values of p/k thus calculated for an infinitely slow rhythm are usually considerably lower than the values for the slowest rhythms experimentally obtained

Table I

1 Experiment	2 Value of s	3 Value of p/k at average rate of discharge	4 Value of p/k at fastest rate of discharge	5 Value of p/k at slowest rate of discharge	6 Value of p/k at an infinitely slow rate of discharge
January 15, 1931	8 35	13 3 (T=0 073)	15 2 (T=0 057)	12 7 (T=0 088)	8 35
January 23, 1931	12 1	16 9 (T=0 067)	21 1 (T=0 043)	14 9 (T=0 095)	12 1
February 18, 1931	13 4	17 4 (T=0 074)	21 3 (T=0 046)	16 3 (T=0 090)	13 4
March 10, 1931	11 8	16 6 (T=0 068)	18 3 (T=0 055)	13 9 (T=0 112)	11 8
May 29, 1931	9 5	13 0 (T=0 091)	16 9 (T=0 052)	10 1 (T=0 178)	9 5
June 17, 1931	9 7	16 3 (T=0 057)	25 2 (T=0 028)	11 2 (T=0 150)	9 7
October 2, 1931	11 4	15 8 (T=0 077)	19 0 (T=0 049)	12 6 (T=0 155)	11 4

(*b*) The curve relating the antecedent and subsequent cycles of premature discharges

Since this curve is usually similar to the single antidromic curve (p 493), it also corresponds closely with the curve of equation (9), for the derivation of this equation depends on the assumption that a reflex discharge has an effect similar to that produced by an antidromic impulse

(*c*) The curve relating the curtailed and subsequent cycles of two antidromic impulses.

A close approximation to the experimental curve for double antidromics has been derived from the single antidromic curve by making two assumptions (pp. 497, 498), both of which are also embodied in those made in deriving equation (9) Since this equation is in close agreement with the single antidromic curve, equations derivable from it must also agree with the double antidromic curves In all such derived curves, however, no account is taken of blocking by absolutely refractory period.

VI SUMMARY

Rhythmic discharge of a single soleus motoneurone has been set up by stimulation of a contralateral afferent nerve The conditions determining the setting up of this rhythmic discharge have been investigated by making a systematic study of the effects produced by an impulse (antidromic impulse) passing up the motor nerve fibre to the motoneurone. Since the rhythmic discharge recommences with an altered phase after interruption by such an antidromic impulse, it is concluded that the rhythmic centre of the motoneurone has been acted on by it

The normal rhythmic discharge is resumed at a longer interval after an antidromic impulse the earlier this acts in a rhythmic cycle. Thus, when the antidromic impulse acts just as a reflex discharge is being set up, the subsequent cycle is no longer than a normal cycle, but, when it acts very early in the cycle, the subsequent cycle is about 1·5 times normal

Under certain conditions a rhythmically discharging motoneurone breaks its rhythm by a premature discharge The cycle following this discharge is lengthened to the same extent as it would be for an antidromic impulse acting at a similar time From this and other evidence it is concluded that an antidromic impulse and a reflex discharge have similar actions on a motoneurone

A second antidromic impulse acting during the cycle subsequent to a previous antidromic impulse is followed by a cycle whose duration depends both on the length of the cycle curtailed by the first antidromic impulse and on the antidromic interval It corresponds closely to that calculated from the action of a single antidromic impulse if it be assumed that the rhythmic centre of a motoneurone is in the same condition at similar intervals before a reflex discharge

Under certain conditions the second antidromic impulse is blocked by an absolutely refractory period set up by the first antidromic. The duration of this refractoriness varies greatly from motoneurone to motoneurone, and also under different conditions in the same motoneurone. It does not seem to exercise any appreciable influence on the rhythmic activity of the motoneurone.

The action of two or more antidromic impulses indicates that there is a basal level below which the rhythmic activity cannot be depressed.

In a general discussion of the experimental observations the events during the rhythmic cycle have been described in terms of the "activity" of the rhythmic centre, by which is meant the propensity of the rhythmic centre to set up a reflex discharge.

Finally a mathematical treatment based on certain assumptions has resulted in equations which are in satisfactory agreement with the experimental observations

We have to thank the Christopher Welch Trustees for defraying the expense of the photographic plates, and one of us (J C E) wishes to express his gratitude to the Medical Research Council for a personal grant

EXPLANATION OF PLATES

PLATE 24.

FIG 2 —Electrical (dark lines) and mechanical (white lines) responses of motor units of soleus muscle in a tetanic crossed extensor reflex In observation 1 only one unit is responding rhythmically, but in observations 2 and 3 there are two units beating with different rhythms Time, 1 d v = 10 σ

FIG 3 —Electrical responses of a single motor unit of soleus muscle during a short crossed extensor reflex evoked by stimulation of the contralateral popliteal nerve during the time shown by the signal

FIG 4 —Rhythmic responses of a single motor unit of soleus muscle interrupted by a single stimulus (antidromic stimulus) applied to the intact motor nerve—shown by the large motor action-current

FIG 5 —As in fig. 4 in another experiment.

FIG 12 —As in fig. 4 but in observations 3 and 4 the cycle following the subsequent cycle of the antidromic impulse is shortened by a premature discharge (*cf* also observation 4, fig 4)

FIG 13.—As in fig 12 but two antidromic impulses have been set up in each observation. In observations 1 and 3 there are two sets of double discharges (*cf* also observation 4, fig 12)

FIG 14 —As in fig. 12 but the premature discharge is so early in observations 1 and 3 that its action current is only visible as a slight alteration of the preceding action current

PLATE 25.

FIG. 17.—Rhythmic responses of a single motor unit of soleus muscle interrupted by two antidromic stimuli 8 σ apart.

FIG. 18.—As in fig. 17 but the antidromic interval is 32 σ.

FIG. 19.—As in fig. 17 but the antidromic interval is 58 σ.

FIG. 20.—As in fig. 18 but in another preparation.

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Infection by Bact. Radicicola *in Relation to the Microchemistry of the Host's Cell Walls.*

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(Communicated by Sir John Russell, F R S.—Received February 19, 1932.)

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[Plate 26]

The intimate relationship of the nodule bacteria to their hosts, the Leguminosæ, offers some fascinating problems in plant chemistry, which have been only partially solved by the researches of the past 50 years. These investigations have been mainly botanical and bacteriological, and such chemical work as there is has been done from an agricultural standpoint, with a view to discovering and explaining the fertilising action of leguminous crops Consequently our hypotheses of the more detailed physiological chemistry of the nodule have been deduced largely from cytological and histological evidence The microchemical mode of attack has been almost entirely neglected.

Even though methods in botanical microchemistry are still very unsatisfactory, it seemed possible that a microchemical study of nodule tissues might throw new light on the nodule problem The results to be reported here are admittedly qualitative and, in some cases, not conclusive, but the author feels that her expectation was justified, and that the field is a fertile one for the future. In general, the methods used were based on those of Molisch (1923) and Tunmann (1913) and on an outline of methods by Dr. Sophia Eckerson (unpublished notes lent by an associate). Additional methods, based on known properties of the substances in question, were devised when necessary.

The work has been done on tissues of lucerne, pea, clover, and broad bean seedlings and nodules Lucerne tissue has been the most extensively used, and unless otherwise indicated, the results described will be those relating to this plant The investigation has been directed along two related lines, which will be considered separately

PART I.—THE INFECTION OF ROOT HAIRS

The Chemical Composition of the Cell Walls of Normal Root Hairs

Despite the importance of the root hairs in the life of the plant, it is surprising that there has been very little work on the chemical composition of their cell walls. Such results as have been published are decidedly contradictory, whether because of actual differences among the plants studied, or because of unreliable methods of testing, it is difficult to say It is not even certain that cellulose is a common constituent of the root-hair wall, Roberts (1916) found it present as an inner layer, but Howe (1921) found none at all and in its place a layer of callose Addoms (1927) obtained uncertain results with cellulose tests.

In the hairs of the leguminous plants here studied, cellulose is undoubtedly present, even to the extreme tips of the hairs It is true that a direct test on fresh tissue, with either chlor-zinc-iodide[*] or the hydrocellulose test[†] reagents, gives a nondescript yellowish green colour, which is inclined to fade within a few hours, although cortical parenchyma, phloem, and even young vascular elements are characteristically blue, as for cellulose But the cellulose tests are strongly positive on root hairs, which have been treated (1) in boiling 5 per cent NaOH or KOH from a half to 6 hours, or (2) in boiling 3 per cent HCl 1 hour followed by boiling 5 per cent KOH 1 hour, or (3) in cold eau de Javelle[‡] 2 to 10 days It will be shown later that these treatments remove an extremely resistant hemicellulose, which is responsible for the failure of the usual cellulose stains to act directly upon root hairs The final and convincing proof of the presence of cellulose in the root hairs will be seen in the following survival and solubility tests :—

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[*] Schultze's reagent from the British Drug Houses was used throughout these tests for the sake of uniformity of reagent.

[†] The hydrocellulose tests were done with I_2KI solution (I_2 0 3 gm., KI 0·75 gm, H_2O 50 c.c) and H_2SO_4 66 or 75 per cent (Eckerson)

[‡] Strasburger formula—20 c.c of 25 per cent. calcium hypochlorite made to 100 c.c with water; 100 c.c. of 15 per cent aqueous K_2CO_3.

1. Tissue treated in boiling 3 per cent HCl 1 hour, followed by boiling 5 per cent KOH 1 hour and examined in water mount, shows root hairs intact, though extremely hyaline They are instantly dissolved in copper-oxide-ammonia reagent* diffused under the coverglass
2. Tissue boiled in 5 per cent KOH alone also shows surviving membranes, soluble in copper-oxide-ammonia
3. Tissue extracted with boiling 0 5 per cent $(NH_4)_2C_2O_4$ 1 to 6 hours, preceded or followed by cold eau de Javelle 2 to 10 days, shows the remainder of the root-hair walls easily soluble in copper-oxide-ammonia
4. Tissue treated with boiling 0·5 per cent $(NH_4)_2C_2O_4$ 1 to 6 hours, followed by boiling 5 per cent KOH 1 hour, contains root hairs easily soluble in copper-oxide-ammonia.
5. Tissue treated in boiling 5 per cent. KOH 1 hour, followed by boiling 3 per cent $H_2C_2O_4$ 1 hour, and either boiling 5 per cent KOH or boiling 5 per cent Na_2CO_3 for 1 hour, shows persisting root hairs easily soluble in copper-oxide-ammonia.

All these methods should remove both pectic and hemicellulosic materials and leave any residual cellulose until the copper-oxide-ammonia treatment These tests have been repeated many times on lucerne, clover, pea, and broad bean tissues, and cellulose has been found consistently even to the tips of both young and old hairs

Of the pectic materials in the root hairs, little can be said Calcium pectate has been shown by its formation of CaC_2O_4 crystals upon treatment with either $(NH_4)_2C_2O_4$ or $H_2C_2O_4$ Roberts and Howe have reported that the calcium pectate forms the outer layer of the wall, so that the crystals in oxalic-acid-treated material would lie in an outer layer of insoluble pectic acid. If this were so in the leguminous plants here studied, the crystals should be liberated by the removal of the pectic acid as, for instance, by formation of its sodium or potassium salt But one can remove all pectic acid by treatment with boiling 5 per cent Na_2CO_3 without disturbing the imbedded crystals, which are, however, set free upon dissolution of the cellulosic residue with copper-oxide-ammonia. This behaviour indicates that the original calcium pectate occurred not in a separate layer but in association with the cellulose-containing layer or layers of the root-hair wall There is evidently some variation in the amount of calcium pectate present, as judged from the relative abundance of crystals. No regularity in this respect could be established, either in relation to the kind of plant or the age and condition of tissue Howe reported pectose "especially found in young tissue" Whether pectose, pectin, or pectic acid is present in the leguminous root hairs cannot be decided from the data available The difficulty arises owing to the simultaneous presence of hemicellulose, which is also stainable by ruthenium red Furthermore, all reagents which remove the hemicellulose also remove the pectic materials, and thus it is not possible to differentiate by subsequent staining

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* Made according to the method in the Eckerson manual from copper filings treated with excess ammonia and allowed to stand open to the air until the reagent is of sufficient strength to dissolve cotton fibres instantaneously The solution deteriorates in 4–6 weeks and must be replaced.

The converse extraction, pectic material from hemicellulose-cellulose residue, is, of course, possible with boiling ammonium oxalate, or oxalic acid followed by such weak alkali as 5 per cent Na_2CO_3, which does not affect the hemicellulose The first hint of the presence of constituents other than cellulose and the pectic substances, in fact came from tests upon root hairs of oxalate-treated material Such root hairs were only poorly stained by Schultze's reagent, but deeply stained by ruthenium red, and not entirely dissolved by copper-oxide-ammonia After treatment with boiling dilute caustic alkali (5 per cent KOH), eau de Javelle in the cold or concentrated NH_4OH in the cold, the offending substance was removed There are several substances which might account for some or all of those properties Suberin, if present, might hinder the cellulose reaction; would be insoluble in copper-oxide-ammonia, but soluble in dilute alkali But the root hairs are not stained by Sudan III or Scharlach R, are soluble in cold 50 per cent chromic acid, and give neither the ceric acid nor phellonic acid tests The failure of Sudan III to stain the hairs eliminates the possibility of there being a fatty substance, as does also the fact that ether extraction failed to remove the resistant substance. Callose would account for the insolubility in copper-oxide-ammonia and perhaps for the failure of the cellulose test, also, Howe reported callose in root hairs of several plants, including the pea and garden bean Her report is based upon positive staining of the root-hair membrane with resorcin blue In the present work no trace of callose could be shown by either resorcin blue or aniline blue staining Nor did the specific solubility tests for callose by cold 1 per cent KOH, $CaCl_2$, $SnCl_2$, or glycerine at 28° C give any indication of its presence

Another class of compounds which might conceivably account for the observed reactions of the root hairs are the proteins. There was indication of their presence in the walls of hairs treated with Millon's, xanthoproteic, or biuret test reagents, but it seemed possible that small quantities might be present and yet escape detection by staining methods. Solubility methods were therefore applied Proteins in general are either acid-soluble or alkali-soluble, whereas the resistant substance of the root hairs survives both prolonged boiling in 3 per cent HCl (1 to 6 hours) and extraction with weak alkalies such as 0·5 to 1 per cent. caustic alkali with boiling for 1 to 2 hours, 5 per cent alkaline carbonate boiling 1 to 2 hours, or the ammonia of copper-oxide-ammonia in the cold for 10 days Furthermore, digestion with proteolytic enzymes, pepsin in 0·2–0 3 per cent HCl, or trypsin at p_H 8 0, fails to remove the inhibitory substance And lastly, the root hairs do not give the Congo red-acid reaction which Priestley and Tupper-Carey (1922) attributed to protein in the walls of root and stem meristems This reaction will be further discussed in relation to the nodule meristem in Part II of this paper

The resistant substance cannot be lignin, since the root hairs, fresh or otherwise, fail to give any of the colour tests for lignin. Neither do they contain pentoses or pentosans in quantity to give the orcinol or phloroglucinol tests. They are also negative to the iodine test for amyloid, applied directly or after treatment of the tissue with eau de Javelle. This is contradictory to the finding of Ziegenspek (1920) who detected amyloid in the tips of the root hairs, which he studied, and considered it to be a transitional product in the formation of cellulose.

The only substances which have been found in the root hairs of the leguminosæ, then, are cellulose, calcium pectate (probably other pectic substances also) and a comparatively resistant material whose properties are as follows solubility in caustic alkali (3–5 per cent.) with boiling 1 hour, in concentrated ammonia and in eau de Javelle slowly in the cold, insolubility in 0·5–1 per cent caustic alkali with boiling ½ to 2 hours; 5 per cent. alkaline carbonate; 3 per cent. hydrochloric acid; 3 per cent. sulphuric acid; 0·5 per cent. ammonium oxalate; 3 per cent. oxalic acid alone or followed by 5 per cent. sodium carbonate, or copper-oxide-ammonia. It is stained by aqueous ruthenium red, iodine green, and some 12 other basic dyes tested; it is stained poorly or not at all by acid dyes. It is coloured yellow by Schultze's reagent, and its presence with cellulose prevents the latter from showing its characteristic blue colour with this reagent. It is not destroyed by heating to 210° or 300° C in pure glycerine, as recommended by Schorger (1926) and Molisch (1923) for distinguishing between hemicellulose and true cellulose. With the last-mentioned exception, the substance has the usual properties of a hemicellulose It has been found in the tips as well as in the lateral walls of the root hairs of the pea, lucerne, broad bean and clover

The Phenomena of Infection

It is a curious fact that infection of a root hair by the nodule organism always occurs at the tip, occasionally at the tip of a lateral branch And yet, so far, it has not been possible to find any chemical difference in the walls at those points, unless it be a slight concentration of the hemicellulose constituent There is, however, some evidence of a physical difference When seedlings of lucerne and clover, grown in petri dishes on the surface of mineral-salts agar, were watered with sterile distilled water, their exposed root hairs swelled out into queer club-shaped and bulbous forms, the tips being first affected When sterile plant nutrient solution was used, deformation of the hairs did not occur. This is in accord with the observations of Stiehr (1903), who reported that the tips of root hairs are first to respond to changes in osmotic relations, and that when hairs are immersed in distilled water, the bursting invariably occurs at their tips It is therefore suggested that the tips, most readily permeable to water, are also most readily penetrated by the bacteria

Many investigators have seen and described the infected root hair, but none has described the exact mechanism of the entrance of the bacteria There would seem to be two possibilities that the bacteria enter mechanically injured or broken root hairs, or that they enter by secreting some substance capable of dissolving the wall at the point of entry Proof of either alternative hinges upon whether the well known curling of an infected hair is induced by the bacteria, or whether the organisms infect a hair deformed from some other cause. It is known that the form of root hairs varies somewhat with the medium in which the plant is grown Roots in moist air generally produce an abundance of straight cylindrical hairs, while those in soil or sand bear hairs more or less distorted by contact with the solid particles. Such contacts might be supposed to produce injuries through which bacteria could enter. To determine whether the bacteria themselves induce a further distortion of the hairs, the numbers of bent and curled hairs on plants in the presence and in the absence of the bacteria were compared. The data shown in Tables I and II are selected from a large number of such counts made upon plants grown in pots of sterilised sand. The presence of the bacteria is associated with a very significant increase in the proportion of bent and curled hairs. In agar-tube cultures, where the factor of mechanical injury is negligible, the results are even more striking, Tables III and IV. The bacteria, therefore, do not rely upon accidentally deformed root hairs but are in some way able to act upon the root hairs Further evidence of this was obtained by the following experiments.

Table I *—Pot culture controls Lucerne, age 3 weeks.

Pot No	Plant	Differential counts of root hairs.			
		Straight	Bent	Curled.	Infected
		per cent	per cent.	per cent	per cent.
1	I	73	27	0	0
	II	78	22	0	0
2	I	74	25	1	0
	II	67	32	1	0
3	I	74	26	0	0
	II	77	22	1	0
4	I	75	25	0	0
	II	83	17	0	0
Mean per cent		75 125	24 5	0 375	0
Standard error		±1 62	±1 55	—	—

Table II —Pot cultures of lucerne inoculated with lucerne bacteria. Age 3 weeks

Pot No.	Plant	Differential counts of root hairs			
		Straight	Bent.	Curled	Infected.†
		per cent.	per cent.	per cent.	per cent.
1	I	41	45	14	2
	II	44	43	13	2
2	I	37	44	19	1
	II	38	48	14	4
3	I	43	46	11	3
	II	39	45	16	3
Mean per cent ..		40 33	45 166	14 5	2 5
Standard error ..		±1·16	±0·71	±1 12	±0·18

† The infected hairs, all being curled, are included in the previous column.

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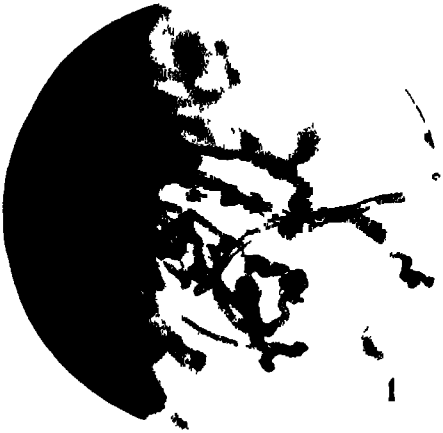
* The percentages in all of the following tables are based upon counts of 20 consecutive hairs at each of five random portions of root from each plant studied.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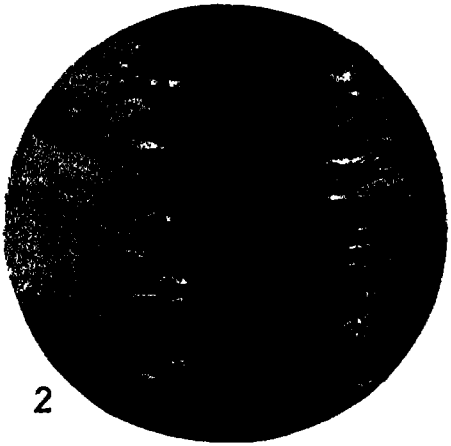
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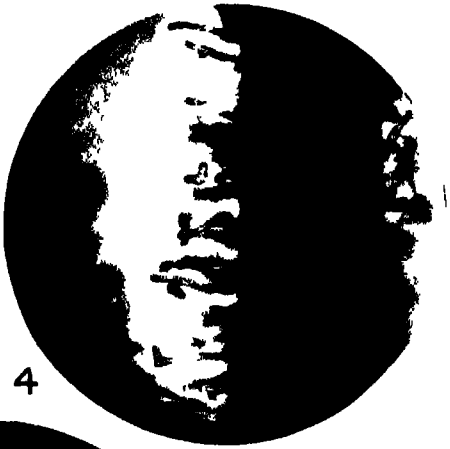
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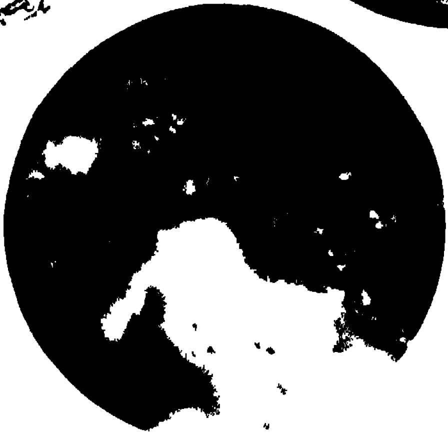
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(*Facing p* 521.)

Table III.—Tube-culture controls. Lucerne, age 3 weeks.

Tube No	Plant	Differential counts of root hairs.			
		Straight	Bent.	Curled.	Infected
		per cent.	per cent.	per cent.	per cent
1	I	92	8	0	0
	II	93	7	0	0
2	I	95	5	0	0
	II	95	5	0	0
Mean per cent		93·75	6·25	0	0
Standard error		±0·75	±0·75	—	—

Table IV.—Tube cultures of lucerne inoculated with lucerne bacteria. Age 3 weeks.

Tube No.	Plant.	Differential counts of root hairs.			
		Straight	Bent.	Curled	Infected
		per cent	per cent	per cent	per cent
1	I	53	38	9	1
	II	52	37	11	0
2	I	57	38	5	0
	II	55	40	5	0
Mean per cent		54·25	38·25	7·5	0·25
Standard error		±1·11	±0·63	±1·5	—

Some 16 cross-inoculation groups among nodule bacteria are known. Bacteria of any one group are not able to form nodules on the plants of any other group. Do the bacteria fail to produce the preliminary curling of the hairs, do they fail to enter the hairs or, once inside, do they find the environment unfavourable? It seemed possible that study of non-infection might lead to a better understanding of infection. Some cross-inoculation experiments were therefore set up in both sand-pot and agar-tube culture, and a study was made of the root-hairs produced. The following cross-inoculations were studied.—

Clover plants × lucerne bacteria.
Lucerne plants × clover bacteria.
Pea plants × clover bacteria.
Pea plants × lucerne bacteria.

The root hairs were carefully examined, but no granules which might be presumed to be bacteria were seen within the hairs. Furthermore, neither infection threads nor abortive nodules were produced by the foreign organisms, but the curling reaction of the root hairs was unusually marked. The hairs were often so crimped, branched, curled, and matted together that it was impossible to count separately those curled at the tips, as if infected, and those otherwise deformed, called bent. Tables V and VI and fig. 1 show results of the pea plant × lucerne bacteria cross. The other crosses gave the same reaction in more or less degree, indicating that the reaction is a general one.

Table V.—Pot-culture controls. Peas, age 1 month

Pot No.	Plant	Differential counts of root hairs.	
		Straight	Bent and curled
		per cent	per cent.
1	I	93	7
	II	86	14
2	I	94	6
	II	90	10
3	I	90	10
	II	88	12
4	I	96	4
	II	91	9
Mean per cent		91	9
Standard error		±1·15	±1·15

Table VI.—Pot cultures of peas inoculated with lucerne bacteria. Age 1 month

Pot No.	Plant	Differential counts of root hairs	
		Straight	Bent and curled *
		per cent	per cent
1	I	27	73
	II	20	80
2	I	26	74
	II	21	79
3	I	28	72
	II	17	83
Mean per cent		23·16	76·83
Standard error		±1·81	±1·81

* Infected hairs, none.

The fact that infection threads were not found in the abnormally curled root hairs does not, of course, prove that the bacteria did not penetrate the cell walls, they might have been killed upon reaching the protoplast Neither does it tell whether the curling reaction is a response to external attack by the bacteria or to some, perhaps toxic, stimulus set up after their entrance To test these possibilities it was decided to do an experiment similar to one reported by Hiltner in 1900 He found that a filtrate of a culture passed through a Chamberland filter exerts a softening effect upon root hairs and causes a typical curvature of them The test was made upon clover and lucerne plants in agar-tube culture Six tubes of each set were treated at the time of planting with 5 c c per tube of filtrates* from 7 day-old cultures as follows —

Clover plants × filtrate of clover bacteria
Clover plants × filtrate of lucerne bacteria
Lucerne plants × filtrate of lucerne bacteria
Lucerne plants × filtrate of clover bacteria

Control tubes were prepared with sterile tap water in simulation of the moisture conditions of the test series Figs 2, 3 and 4, Plate 26, are photomicrographs of the results, taken when the plants were 3 weeks old Curling as well as other deformation of the root hairs, including branching (note especially fig 4), were produced in the absence of the bacterial cells The results indicate that the curling which normally precedes infection of the root hairs is produced by a substance secreted by the bacteria before they enter

These root hairs curled by bacteria-free filtrates, those curled by bacteria of other cross-inoculation groups, and hairs actually containing infection threads were repeatedly tested for the constituents of the normal root-hair wall, namely cellulose, pectic materials (especially calcium pectate) and the resistant hemicellulose All were found, even in the curled tips There was absolutely no indication that the bacterial action had produced any chemical change in the walls This raises the question as to how the bacteria penetrate the walls of the root hair

Since cellulose is present in the root-hair wall, cultures of the bacteria were tested upon various cellulose media lucerne root-extract agar containing ground filter paper, similar agar with strips of sterile filter paper laid upon the surface of the agar and inoculated by streaking across the strip so as to insure direct contact of the bacteria with the cellulose; similar agar containing native cellulose prepared from lucerne root-meal, and all of the above media with 0·25 per cent. of sucrose to ensure initial growth of the bacteria. In no case was there any visible attack on the surrounding cellulose nor any indication of attack even close to or under the line of growth, when the plates were tested for cellulose by the hydrocellulose and chlor-zinc-iodide tests.* These findings agree with Beijerinck's original report (1888) on the absence of any cellulose-dissolving enzyme in his *B. radicicola*.

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* The filtrates were prepared from suspensions of the bacteria in sterile tap water by Berkefeld filtration, and were proved bacteria-free by test of 1 c.c portions on slopes of root-extract agar and by the fact that neither infection threads nor nodules were formed on the test plants of homologous group.

The action of the nodule bacteria on other constituents of the root hairs, such as the pectic materials, remained to be investigated, since no previous report on the subject could be found. Root extract media were prepared (1) with no added carbohydrate, (2) with 0·5 per cent. of apple pectin,† (3) with apple pectin plus 0·5 per cent. of sucrose, (4) with 0·5 per cent. of lemon pectin,‡ (5) with lemon pectin plus sucrose, (6) with 0·5 per cent. calcium pectate,§ and (7) with calcium pectate plus sucrose. The pectins were sterilised separately in distilled water solution and were added aseptically to the media as required. The p_H of the finished media ranged from 6·8 to 7·2. In all, 17 cultures of the nodule bacteria representing various cross-inoculation groups were tested on the pectic media. Whenever sucrose was present, abundant moist growth, characteristic of the cultures used, was obtained. In the controls with no added carbohydrate, growth was exceedingly scanty (only nutrients of the root extract being available, and in these pectic test media only one-half of the strength of root extract ordinarily used in the Rothamsted Laboratory had been used). In the plain pectic media, growth was no better than in the controls, and there was no indication of utilisation of the pectins or calcium pectate.

It would be interesting to try the effect of the bacteria on the hemicellulose constituent of the root-hair walls, but so far it has not been possible to isolate the specific hemicellulose in anything like sufficient quantity for the preparation of media. On the other hand, there is little likelihood that the bacteria would be able to destroy it in culture, when they apparently do not do so in the root hair itself. Whether the root hair wall is destroyed at the exact point of the bacteria, or whether it is re-formed there after invasion, are points too fine to be settled by direct observation.

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* These tests were made on the agar films dried in an oven at 100° C.

† From the British Drug Houses.

‡ From the California Fruit Growers' Exchange Laboratory.

§ Prepared from the lemon pectin.

A study of the root hair infection thus leads to the following conclusions —

(1) The bacteria do not enter mechanically injured root hair tips,

(2) they have not been shown capable of dissolving the known constituents of the root hair walls, but

(3) they do produce a secretion capable of modifying the wall in some way, as evidenced by abnormal curling of the root-hair tip either in the presence of the bacteria or of their fresh metabolic products

It is further suggested that the bacteria attack the tip of the root hair because of some pre-existing physical difference at that point, as evidenced by its susceptibility to osmotic change

There is curious limitation of the infection of the curled and bent root hairs The great majority are not visibly infected. Thornton (1929) has reported 4 per cent. infected root hairs for lucerne seedlings and the various counts made in the present study have ranged from 2·4 to 5·5 per cent There is also some limitation of the number of infections which develop into nodules Few counts of this phase of the problem have been made, but it seems of interest to present one set of figures relating to one of the lucerne plants studied .—

Total number of hairs on 1 inch of root	1121
Number of infected hairs in above section	62
Percentage of hairs infected	5·5
Number of nodules on the plant	5
*Estimated total length of root system (inches)	5·5
Estimated total number of hairs	6165
Estimated total number of hairs infected	341

The plant was approximately 6 weeks old, its five nodules were well grown and apparently no new nodules were forming Consequently it appears that only 1 in 68 of the original infections succeeded in producing a nodule The other infections apparently stopped growing, many with the infection thread only part way down the root hair.

PART II —THE INFECTION THREAD WITHIN THE NODULE

Any attempt to explain the invasive powers of the nodule bacteria must account for their original penetration of the root hairs and also for the spread of infection through the cortical tissues of the root and, later, throughout the

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* At the time of the count bearing or having borne root hairs.

growing nodule Many of the histological studies on root nodules have referred to the infection threads as "passing from cell to cell" and thereby extending the infection Recently Milovidov (1926, 1928) has found that infection in the lupin is spread through active division of the host cells, but it is admitted that the lupin is exceptional in this respect and that in the majority of Leguminosæ the infection threads are active. It remains, then, to show how the infection threads cross cell walls

As in Part I of this report, the microchemical investigation was begun by a study of the normal constituents of the parenchymatous tissue of the root cortex and of the nodule Briefly it may be said that the methods used were the same as those described for the study of root hairs, and that the following constituents were found cellulose, calcium pectate and probably pectose, and a hemicellulose less resistant than that of the root-hair wall (see p. 515) The hemicellulose of the parenchymatous tissue is, for instance, destroyed by heating to 300° C in pure glycerine or boiling 1 hour in 5 per cent KOH; is not destroyed by boiling 1 hour in 3 per cent HCl, but is destroyed by subsequent heating 1 hour in 5 per cent Na_2CO_3 or 0 5 per cent KOH for ½ hour to 1 hour, is destroyed by 6 hours' boiling in 3 per cent HCl alone, but not by 0·5 to 2 per cent. KOH 1 to 2 hours nor for 6 hours in 0 5 per cent. $(NH_4)_2C_2O_4$, it is not soluble in copper-oxide-ammonia or concentrated ammonia in the cold, but is slowly soluble in eau de Javelle in the cold It does not interfere with the chlor-zinc-iodide test for cellulose, it is stainable by ruthenium red and therefore interferes to some extent in the study of the pectic substances in the nodule It is stained slightly or not at all by iodine green.

In an elongated nodule of the lucerne type, for instance, the distal end contains the meristematic tissue, and the main body of the nodule, as one passes toward the proximal or basal end, is composed of progressively older tissue. Thus the tissue is graded from the young and meristematic to the old and mature, and one would expect the composition of the walls to vary accordingly In the following discussion only the meristematic tip and parenchymatous tissue of the mature bacteroid area are discussed. The walls in question are very thin, and it is not always possible to differentiate between substances in the middle lamellæ and secondary layers of the walls

Priestley and Tupper-Carey (1922) and Tupper-Carey and Priestley (1924) have studied the difference in composition of the meristematic and mature walls of root and stem tips and have reported that a Congo red-acid reaction reveals one striking difference Meristematic walls, once stained by aqueous Congo red, remain red in the presence of 10 per cent acetic acid, because of the specific isoelectric point of the protein which they contain. Mature walls, devoid of protein, are turned blue by this acid The reaction was highly successful when applied to longitudinal sections of fresh nodule tissue, it showed distinctly red walls in the meristematic cap, grading to blue walls in the older bacteroid area Other sections of the same nodule were held over 24 hours at 37° C in a peptic-digest bath containing 0·3 per cent HCl, and then tested with the Congo red-acid reaction This time the meristematic walls also were blue, indicating that the proteolytic enzyme had removed the substance responsible for the original red staining Moreover, the bacterial bodies which previously gave the protein reaction were much shrivelled by the peptic digestion, and their residues were thereafter blue in the Congo red-acid test

In its pectic and hemicellulosic content also, the meristematic cap differs from the old tissue The oxalate test for calcium pectate, for instance, shows well-formed calcium oxalate crystals in the older tissue and absolutely none in the extreme meristematic tip But there is some form of pectic material in the tip, as evidenced by the following observations Ruthenium red colours fresh tissue pink throughout the bacteroid and meristematic areas, indicating either hemicellulose or pectic material or both Copper-oxide-ammonia at this stage fails to attack the tissue Other tissue boiled 6 hours in 0·5 per cent $(NH_4)_2C_2O_4$ shows older parts of the tissue still pink in ruthenium red, but the meristematic tip very pale or colourless Copper-oxide-ammonia at this stage violently attacks the meristematic tip and dissolves its cell walls, while causing only collapse in the older tissues. These effects indicate pectic material throughout the tissue but little or no hemicellulose in the extreme meristematic tip And since the pectic material in the tip fails to give the calcium pectate test, it may be assumed to be pectose or pectin.

Solubility and staining reactions showed the presence of cellulose in both young and old walls The colour tests were particularly interesting because they revealed certain clustered patches devoid of cellulose Fig. 5 is a photomicrograph of cells in the bacteroid area of a lucerne nodule stained with Schultze's reagent. Similar pits were found in the cells of the pea and clover nodules and the normal parenchyma of their root cortices as well. The patches were first seen in tissue stained with either Schultze's or the hydrocellulose test reagents (best in tissue previously treated with alkali) but are also visible in fresh tissue deeply stained with methylene blue or Congo red One's first impression on seeing such preparations is that the patches are actual perforations in the walls, perhaps channels of the plasmodesmen

Gardiner (1897, 1901) who studied plasmodesmen, found them in parenchyma tissue and in his 1901 paper made the following suggestive remark "Parenchyma cells of the pith, if mounted in weak iodine containing about 10 per cent. sulphuric acid, show the cellulose walls blue excepting where the closing membrane is stained yellow" That there is actually a closing membrane across the patches in the nodule cell walls seems probable from the following observation Some tissue boiled 1 hour in 3 per cent HCl and stained with ruthenium red, showed very thin and delicate cell walls of the bacteroid-tissue, coloured uniformly red, apparently only the middle lamellæ being stained Schultze's reagent, diffused under the cover-glass, revealed doubleness of the walls, which appeared much thicker than when stained with ruthenium red alone Also the characteristic white patches appeared *in the thick blue layers of the walls* Some pieces of tissue observed on edge showed a definitely pitted effect And so, while the patches are evidently similar to those seen by Gardiner in the walls of parenchyma, they should rather be interpreted as pits in the secondary layers of the walls and not necessarily protoplasmic connections, as the plasmodesmen are now conceived to be They may, of course, be the remains of the original plasmodesmen, bridged over by the middle lamellæ but with the perforations of the secondary layers of the walls enlarged and distorted by the stresses of growth However that may be, the presence of these differentiated patches in the walls is most significant They vary in size and shape, as may be seen from the photograph On tissue stained with Schultze's reagent, the diameters ranged from 2·5 to 8 micra ; this tissue was, of course, somewhat swollen by the reagent, so that the measurements are not to be taken as exact, but they indicate that the pits are of sufficient size to admit small infection threads This is not a new suggestion A search of the literature showed that Vuillemin (1888) also found the patches not stainable by chlor-zinc-iodide He described them as "*fenêtres*" and ingeniously suggested that, thanks to them, the walls are readily permeable yet maintain their structure and strength

The nature of the infection thread must now be considered in order that its behaviour as it crosses a cell wall may be understood This is an old and much debated problem in the cytology of the nodule It has been known since the early descriptions by Eriksson (1873) and Ward (1887) that the infection thread spreads out into a funnel-shaped enlargement as it crosses the wall Ward (1888) attached no importance to the enlargement, remarking that the expansion of the cell wall after the passage of the infection thread would account for such an experience Prazmowski (1890) described the enlargement as a passive spreading of the thread in the intercellular space, formed by the splitting apart of the middle lamella under the boring action of the infection thread On the nature of the infection thread itself, opinion is divided between two opposing views, namely, (1) that the thread is a zoogloeal strand imbedding the bacteria, or (2) that it is a definite tube in which the bacteria lie imbedded in their own slime Several of the early investigators, Eriksson (1873), Ward (1887), Vuillemin (1888), Koch (1890), and Laurent (1891) reported the outer sheath of the infection thread to contain cellulose Moeller (1892) and Schneider (1892) granted that such a membrane is present but held it to be a deposit by the host cell to wall off infection Kny (1879), Prillieux (1879 and 1890), and Zukal (1897) found no membrane and therefore considered the threads to be plasmodia Dawson (1900) failed to confirm the early reports of a cellulose membrane but recognised that the infection thread is tube-like and bounded by a definite sheath Peirce (1902) and Fred (1909) referred to the threads as zoogloeal strands, and Burrill and Hansen (1917) remarked their "solid hypha-like structure bearing remarkable resemblance at times to a tube"

Anyone who studies the infection threads carefully must be impressed by their great variety of form, yet definiteness of boundary, and with the sharp contrast in refractility between them and the protoplasm of the host cells This is particularly evident in the infection threads in the root hairs, examined fresh in a water mount Often in such mounts some infected hairs will be found mechanically broken, and then one may see the infection thread projecting from the open end, as rigid and clear as a glass rod Or perhaps the lateral walls of the hairs will be broken away and the infection thread, still intact, may be seen holding the broken hair in place A gentle tap on the cover-glass will prove how stiff and resistant is such an infection thread. It is hard to imagine a zoogloeal strand behaving so Moreover, in prepared slides it is common to see an outer layer or sheath which is definitely stained by the same dyes that affect the cell walls of the plant Sometimes, however, the sheath may be very poorly developed or not even present These naked threads are usually seen in very young infection spots or in cells apparently newly invaded

A microchemical study of the sheaths of infection threads was made in order to discover their probable origin. They were first tested for cellulose, which was considered to be present on the basis of the following observations —

1. Sheaths survive—

(a) Boiling 6 hours in 3 per cent HCl

(*b*) Boiling 1 hour in 3 per cent. HCl or $H_2C_2O_4$, followed by 1 hour in 5 per cent KOH

(*c*) Heating to 300° C. in glycerine (alone or after pre-treatment with 3 per cent boiling HCl 1 hour and 5 per cent boiling KOH 1 hour)

(*d*) Boiling 0·5 per cent $(NH_4)_2C_2O_4$ 1 to 6 hours

(*e*) Heating to 180° C in 50 per cent KOH

2 Sheaths in tissue treated as in (*a*), (*b*) and (*c*) above instantly soluble in copper-oxide-ammonia

3 Sheaths in tissue after any of the above treatments show positive staining with Schultze's or the hydrocellulose test reagents Fresh tissue also gives a positive test for cellulose but not so deep a colour

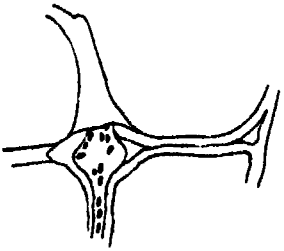

Fig 1—A camera lucida drawing of the sheath of an infection thread as it approaches the cell wall

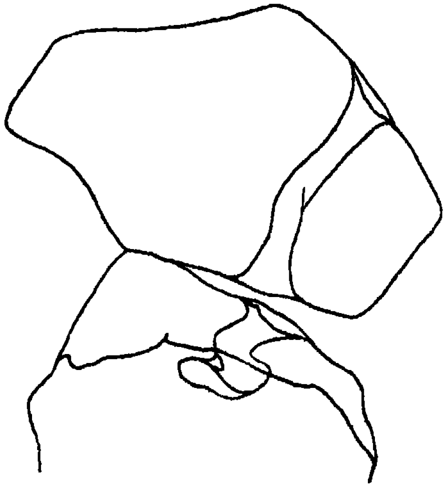

Fig 2—A camera lucida drawing showing more clearly the separation of the host cells and the absence of sheath across the middle lamella.

The colour tests for cellulose revealed an interesting and significant fact about the infection thread Tissue treated to remove pectic and hemicellulosic materials is necessarily fragile and easily macerated, owing to loss of its "binding" materials The cellulose tests on such material produce a certain swelling which tends to separate the cells and shows that *there is no sheath around the infection thread as it crosses the middle lamella* Text-figs 1 and 2 are camera lucida sketches of such pieces of infection thread In one test on tissue previously treated for 10 days in eau de Javelle in the cold and boiled 1 hour in 0·5 per cent $(NH_4)_2C_2O_4$, the cells actually broke apart and floated away in the currents under the cover-glass Several were observed in which the sheaths of the infection threads appeared as complete tunnels through the cells Such observations provide strong evidence that the sheath is a deposit of the individual plant cell and not of the bacterial strand Also the sheath is obviously attached to the plant cell wall through the "funnel-shaped enlargement", it is of the identical shade of blue in the cellulose-tested material and merges imperceptibly into the lateral walls of the cell The expansion of the slime thread lies entirely within the middle lamella, as Prazmowski pointed out

The sheath also contains other constituents which relate it to the plant wall. Material extracted for 10 days in copper-oxide-ammonia and no longer giving the cellulose reaction, still contains the sheaths of its infection threads These sheaths are stainable with ruthenium red and thus may be either pectic or hemicellulosic That they are not composed of calcium pectate is shown by negative oxalate tests They do contain the hemicellulose of the plant cell wall as shown by their survival of 6 hours extraction with 0·5 per cent $(NH_4)_2C_2O_4$, after which they can still be stained by ruthenium red but not by iodine green They respond also to other properties of the hemicellulose noted earlier in this paper in connection with the study of the walls of the bacteroid cells The presence of the hemicellulose in the sheaths interferes with the detection of such pectic substances as pectose or pectin

There is one other line of evidence that may be cited in favour of the host plant origin of the infection-thread sheaths In material which has been heated to 300° C in pure glycerine, one often finds diamond-shaped pieces of cellulosic infection-thread sheath attached to some of the cell walls This indicates that the sheaths form first at the cell walls, and would suggest that they originate at the cell walls and not along the gummy matrix of the thread, which extends through the cell as well as across its boundaries

SUMMARY AND CONCLUSIONS

Part I —The Infection of Root Hairs

(1) It is statistically proved that infection of the root hairs is not a mere invasion of mechanically injured or broken root hairs The presence of the bacteria, even of strains belonging to foreign inoculation-groups, causes a significant increase in the number of curled and bent hairs

(2) The nodule bacteria invariably attack the tip of a root hair, a fact which may be related to some pre-existing physical difference at that point

(3) The bacteria produce a secretion capable of modifying the wall, as evidenced by the abnormal curling of the root-hair tip This secretion is separable from the cells by filtration and is not specific for the plants of the cross-inoculation group to which the bacteria belong.

(4) The bacteria in culture could not be shown to attack cellulose, pectin or calcium pectate Curled tips of root hairs, whether or not infected, contain the same constituents as normal hairs These constituents are cellulose, calcium pectate and probably pectose, and a very resistant hemicellulose

Part II —The Infection Thread within the Nodule

(5) The cell walls of the nodule contain cellulose, a hemicellulose, calcium pectate in the mature parts, and pectose at least in the meristematic tip. Walls of the tip also give a protein reaction

(6) There are numerous pits perforating the secondary layers of the walls, but the middle lamellæ appear to be continuous These pits are of sufficient size to admit infection threads

(7) The infection thread is surrounded by a definite sheath consisting of cellulose and hemicellulose, calcium pectate is absent and the presence of other pectic materials has not been confirmed

(8) The sheath does not cross the middle lamella, and is probably a deposit of the individual plant cell

(9) It is suggested that the bacterial zoogloea crosses a cell wall by way of the pits and is later covered by the sheath, a product of host cell activity

Acknowledgment

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EXPLANATION OF FIGURES.

PLATE 26.

FIG. 1.—A photomicrograph of root hairs of a pea plant grown in the presence of lucerne bacteria.

FIG. 2.—A photomicrograph of normal root hairs of a clover plant.

Fig. 3.—A photomicrograph of root hairs of a clover plant grown in the presence of a filtrate of lucerne bacteria

Fig 4.—A photomicrograph of root hairs of a lucerne plant grown in the presence of a filtrate of clover bacteria.

Fig. 5 —A photomicrograph of the walls in the bacteroid area of a lucerne nodule stained with Schultze's reagent The pits in the secondary layers of the walls are visible as white patches

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Francis Darwin

FRANCIS DARWIN—1848-1925

Francis Darwin, the third son of Charles Darwin, was born at Down on August 16, 1848, he died at Cambridge on September 19, 1925. In his 'Recollections' (one of the essays in "Spring-time and other Essays" (1920)) he says that he was christened at Malvern—"a fact in which I had a certain unaccountable pride But now my only sensation is one of surprise at having been christened at all, and a wish that I had received some other name" When he was twelve years old he went to the Grammar School at Clapham kept by the Rev Charles Pritchard, who became Savilian Professor of Astronomy at Oxford. This school was selected on account of its nearness to Down, and also because it "had the merit of giving more mathematics and science than could then be found in public schools" He was admitted to Trinity College, Cambridge, in 1866, where, in those more peaceful days, from his bedroom he heard the nightingales sing through the happy May nights He described the teaching of biology at Cambridge as being "in a somewhat dead condition Indeed, I hardly think it had advanced much from the state of things which existed in 1828, when my father entered Christ's College The want of organised practical work in Zoology was perhaps a blessing in disguise, for it led me to struggle with the subject by myself. I used to get snails and slugs and dissect their dead bodies, comparing my results with books hunted up in the University Library, and this was a real bit of education" On one occasion "a thoughtful brother sent me a dead porpoise, which (to the best of my belief) I dissected, to the horror of the bedmaker, in my College rooms"

After obtaining a First Class in the Natural Sciences Tripos in 1870 he went to St. George's Hospital and in due course took the Cambridge M B degree In London he "had the luck to work in the laboratory of Dr. Klein," who gave him "the first opportunity of seeing science in the making—of seeing research from the inside" and thus implanted in his mind the desire to work at science for its own sake The chance of doing this, he says, came when his father took him as his assistant He did not carry out his intention of becoming a practising physician · "happily for me the Fates willed otherwise" He returned from London to the home at Down and for eight years acted as secretary and assistant to his father.

On Charles Darwin's death in 1882 Francis went to live at Cambridge and devoted himself to Botany. He was elected a Fellow of Christ's, his father's College, and in 1884, having been appointed University Lecturer in Botany, he co-operated with the Reader in Botany, Dr. Vines, in teaching plant physiology. On the appointment of Dr Vines to the Chair of Botany at Oxford Darwin became Reader and held that position until 1904. From 1892 to 1895 he acted as Deputy to Professor Babington, who had long ceased to take any part in the modernised and to him distasteful methods of instruction Before coming to Cambridge Darwin spent a few months in 1878 in the famous laboratory at Würzburg under Professor Sachs In later years his relationship with Sachs came to an unhappy ending, he published a paper containing some criticisms of the master's researches Having written to Sachs and obtained no reply, he travelled to Würzburg in order to ascertain the position of affairs On asking Sachs why he was angry with him he received the reply "the reason is very simple; you know nothing of Botany and you dare to criticise a man like me" In 1881 Darwin spent a short time working with Professor de Bary at Strassburg

In 1882 he was elected Fellow of the Royal Society, he was Foreign Secretary from 1903 to 1909, and Vice-President 1907–1908 In 1909 the Honorary Degree of Doctor of Science was conferred upon him by his own University at the Darwin Centenary, he was the only Englishman so honoured. "I have been astounded and delighted," he wrote in a letter, "at a most charming letter from the Vice-Chancellor asking if I would have a Sc D at Cambridge Of course I can only accept gratefully I hope you approve—it is against the principle of no Englishman need apply" Reference may be made here to his activity in connection with a small volume presented by the Syndics of the Cambridge University Press to delegates at the Centenary celebrations, entitled "The Foundations of the Origin of Species, a Sketch written in 1842" The manuscript of this sketch is referred to by Charles Darwin in his autobiography as "a very brief abstract of my theory", it was discovered in a cupboard at Down after Mrs Darwin's death in 1896, and was edited with an introduction by Francis Darwin for distribution at the Cambridge celebrations

He was an Honorary Doctor of Science of Dublin, Liverpool, Sheffield, and Brussels, an Honorary Doctor of Laws of St Andrews and an Honorary Doctor of Philosophy of Upsala and Prague In 1908 he was President of the British Association at the Dublin meeting, an appointment which gave general satisfaction to his colleagues both on personal grounds and as a recognition of the claims of botany after a lapse of forty years In 1912 he was awarded the Darwin Medal, in 1913 he was knighted

Darwin was married in 1874 to Amy Ruck of Pantlludw, North Wales. She died in 1876 in giving birth to their son, Bernard Darwin Many years later (1920) he wrote "The Story of a Childhood" (privately printed) which is made up of extracts of letters (1877–1891) written to the mother of his first wife, the story is of the early years of Bernard Darwin. This collection of excerpts is evidence of his love of home, of the sincerity of his statement that "the domestic and intimate parts of life are the most lastingly happy", and of his faculty of winning the confidence and love of young children. In 1886, with true prophetic vision, he wrote to Mrs. Ruck, when his son was ten years old, "I am sure Bernard will be a great swell at golf some day"

In 1883 he married Ellen Wordsworth Crofts, lecturer at Newnham College They lived at Wychfield, which they built in the grounds of his mother's house at Cambridge, until his wife's death in 1903 In these peaceful years he did the best of his work, and her powers of encouragement and criticism greatly helped him in the writing of his father's life They had one daughter, Frances, now the wife of Professor F. M Cornford, of Cambridge

In 1913 Darwin married the widow of Professor F W Maitland, who died in 1920

It is fitting that the best literary production of Francis Darwin should be the "Life and Letters of Charles Darwin" As Sir Charles Sherrington said in his presidential address to the Royal Society in 1925, this book is "by common consent one of the most admirable and delightful accounts ever written of a great scientific life, the modesty and simplicity of the presentation contributing to its charm" He gave a true picture of his father's personality, his love of animals, his high sense of honour, his thought for those who served him, however humble their position, and his modesty These are some of the many qualities inherited by Francis and illustrated in the two volumes of essays, "Rustic Sounds" (1917) and "Springtime and other Essays" (1920) As a reviewer of one of the volumes says "a certain note of intimacy seems to run through the whole" In an essay entitled "Recollections," included in the later series, there are many delightful sketches of scenes and events which remained fresh in his memory He recalls a general impression of "unwillingly attending divine service for many boyish years" and the "singular custom" at Down of having family prayers on Sunday only, the butler Parslow, who was "a kind friend to us all our lives" and had "what may be called a baronial nature" Darwin was a lover of music, though, as in other things and persons, his tastes were eclectic, as a performer he preferred the bassoon, the flute and the recorder, "one of the most ancient of wind instruments," which he described with other old instruments in one of his essays

Darwin was a genuine lover of Nature, his sister, Mrs Litchfield, spoke of him as "the only one of my father's children with a strong taste for natural history" An essay on "A Lane in the Cotswolds," written when he lived at Brookthorpe, reminds one of W H Hudson The following sentence recalls the poet's words "The miracle of an earth reclad" "The great revolution that breaks out in the spring, when the store-houses of the plant pour nutriment into the numberless awakening buds, is a miracle annually repeated in the endless procession of life" In later life he amused himself by following the example of Leonard Blomefield, whose "Naturalist's Calendar" he edited in 1903, and other naturalists in noting the dates of the first blooms of wild plants After giving a list of flowers seen in February and March he says "To a lover of plants, this commonplace list will, I hope, be what a score is to a musician, and will recall to him some of the charm of the orchestra of living beauty that springtime awakens"

In general literature he was faithful to a few authors, among his favourites were Dickens, Jane Austen—in whom he took a special delight—"the power of endlessly re-reading the works of Jane Austen is the only advantage conferred by a bad memory"—Trollope, Thackeray, Walter Scott, Mrs. Gaskell, George Eliot—especially Middlemarch—and other, earlier writers

I often recall what was probably the first occasion, now nearly fifty years ago, on which I spoke to Francis Darwin At the end of one of his lectures I asked him a question to which there was no doubt in my mind an answer would at once be given, he answered me with a smile, 'I have not the slightest idea" This was thoroughly characteristic of him, many men would have admitted their ignorance to an undergraduate less directly, if indeed at all, but he was always absolutely straightforward and made his students feel that he was a fellow learner Neither by manner nor speech did he ever act the part of a superior person In an address on "The Teaching of Science" he said, "The only lectures which impressed me, as an undergraduate at Cambridge, were those of the late Sir George Humphry, and his most striking words were confessions of complete ignorance about many parts of physiology" As a lecturer he made no attempt to acquire the art of impressing an audience by devices familiar to many speakers unconsciously he made an appeal by his naturalness and simple directness He had something to say and said it in the simplest way On my asking him how he had enjoyed a certain public lecture, he replied in some such words as these "The place where I lectured was separated from the rest of the large hall by a curtain, from time to time some wretched person pulled back the curtain to see what was going on, and at once retired, obviously saying to himself, 'Good God! here's a lecture'"

It was a privilege to be associated with Darwin in the preparation of the "More Letters of Charles Darwin" (1903), his reminiscences of the family life at Down, descriptions of this and that "tiresome person," his keen sense of humour, were a constant joy

The photograph accompanying this Notice was chosen because it shows Darwin with one of many successive companions (Scrubbins) from whom he was seldom separated His dogs were treated as he treated his best human friends and he seemed to derive no little satisfaction from their companionship, possibly for the reason that they never bored him As a quotation at the head of an essay on "Dogs and Dog Lovers" he chose Archbishop Whately's words—"The more I see of men, the more I like dogs." The dog he liked best was "an inferior Irish terrier who gave me much trouble and anxiety."

Darwin always found it very difficult to hide his feelings when in uncongenial company; he had strong prejudices, quickly formed, and was not reticent in expressing candid and terse opinions Of him it was true to say

> "He hath a heart as sound as a bell, and his tongue is the
> Clapper, for what his heart thinks his tongue speaks"

Intolerant of any trace of artificiality and affectation, he was quick to blame and no less quick to praise He realised more than most men the value to younger people of words of encouragement and approval; no one could give greater pleasure than he did by his outspoken words of affectionate regard, words that went to the heart because they came from the heart His path through life was smooth; his hours of leisure were not curtailed through the necessity of adding to income by any form of drudgery He was conscientious in the discharge of such duties as he undertook, but he did not willingly take upon himself tasks which made large demands upon his time, or brought him into contact with public business He was generous in deed and thought to those less comfortably placed, and brightened many lives by his unfailing kindness and sympathetic encouragement He was a man who inspired affection; his kindly blue eyes, his short calls in the middle of a morning's work in the laboratory, with apologies for dropping in with nothing particular to say, his sense of humour—humour which, as he said in speaking of Francis Galton, is "so priceless an antiseptic to sentimentality"—his exceptionally lovable personality, are memories which do not fade

A C S

Some presentation may now be made, by another hand, of the scientific work that Francis Darwin achieved His publications, dated from 1872 to 1921, included, beside several scientific books, some sixty papers in addition to lectures and addresses No attempt will be made to survey all these, and this notice only deals with the two main chapters of his biological activity For each of these, a sketch of the subject is given with Darwin's particular contributions in the foreground, set against a background of the general position of that chapter of plant physiology The two sketches are followed by a few notes mentioning some of his other larger publications

I—FRANCIS DARWIN'S PART IN THE INVESTIGATION OF GROWTH-CURVATURES

By growth-curvatures are understood those movements that growing parts of plants make in response to the directive influence of factors of the environment, such as unequal lateral illumination or unnatural relations to the direction of gravitation Outstanding among the curvatures thus engendered are those labelled positive or negative geotropism and positive or negative heliotropism. From the close study of these phenomena during the last half-century there has arisen a whole new world of vital relations and theories The earlier days of this human struggle to create a science we have now to survey

Between 1880 and 1908 Darwin published a dozen papers and some public lectures on growth-curvatures, and gave in addition three presidential addresses to the British Association In this way he came to be identified in England with this field of work When he started his independent work the times were singularly apt for him, so we may begin with a brief statement of the influences, Continental and English which combined in the years round about 1881 to produce a blossoming time that justified a text-book of that date appearing with the title "The New Botany" The essential forces at work were those that created plant-physiology abroad and plant-biology at home Both of these found a susceptible disciple in Francis Darwin

In the years just before 1879 Sachs of Wurzburg had carried out a series of studies of the anisotropic behaviour of plants– a term including the lateral curvatures by unequal growth already mentioned, as well as a variety of other aspects of unilateral organisation First for geotropic curvature, and later for heliotropic also, he came to the conclusion that these reactions were not engendered by direct control of the external conditions but belonged to the chapter of physiology which centred in "Irritability"—the response to stimuli He was struck by the similarity of these responses of plant-movement with the motor responses of animals, and he held that the sensitiveness of the plant, or of its parts, to one kind of stimulus in one region and to another kind in another region was a general biological principle for plants, analogous with the provision of sense-organs in animals

Sachs was a brilliant experimenter—"the father of experimental plant physiology" and invented most of the simple methods still used for demonstrating the reality of the physiological attributes of the living plant.

The year 1881 was marked out by a great botanic event It was then that Plant-Physiology may be said to have crystallised out in its pure form from the general solution of mixed botanical knowledge In that year W. Pfeffer published the first issue of his masterly treatise in which the subject was given its general set of primary concepts, arranged in the clear philosophic relation that was to determine the facies and articulation of the subject for the next 50 years In no part of his text-book was the treatment more brilliant than in his presentation of Irritability

Turning to our own country at the same epoch, we find the outstanding figure of Charles Darwin who had been spending a succession of years in the detailed study of the biological behaviour of plants. His volumes on fertilisation and on climbing plants were followed in 1880 by one entitled "The Power of Movement in Plants" All this extensive work was based on the outlook and problems that arose from his previous survey of Natural Selection. For each exploration Charles Darwin studied the life of a great variety of plants and made out the agreements and differences in the domestic economy of their individual lives Such work was a blend of biology and physiology and has been labelled ecology, earning for its inventor the style of the 'father of plant ecology"

Having sketched the giant scientific forces that were then energising the study of plants we note that in 1874 a young man of 26 about to take the M B degree at Cambridge, started his scientific career by assisting his father with experimental work at Down, in preparation for the volume that was planned on the movements of plants When this appeared, completed, in 1880 the title page bore the inscription "by Charles Darwin assisted by Francis Darwin"

Throughout his life Francis Darwin had a profound belief in the philosophical identity of inheritance, memory, habit and the mnemonic association of stimuli He never declared whether the parental influence came by inheritance or as an external stimulus during this apprenticeship, but his career shows that the lines of thought and of experimentation then initiated became a deep-seated habit for the rest of his life For a short time during this period of work with his father, Francis left Down and went on a pilgrimage to the laboratory of Sachs at Würzburg where he made daily contact with that brilliant autocrat and with the current work in physiology His scientific personality now took its final shape and when his father died in 1882 he was launched on an independent career as a talented experimental plant physiologist whose mental interest in his work was strongest on the recurrent question of what might be the biological origin and significance in the life of these plants of the particular attribute under investigation.

Having sketched the development of the worker, we pass to consider the work, and we may start by stating the general outlook on the nature of Growth-Curvatures that was coming to be adopted about 1880

Heliotropic curvatures under lateral illumination had been regarded by De Candolle as just the differential outcome of strong light on one side and weak light on the other side, determining different local rates of growth on the sides with the inevitable result of curvature The investigators of the period we are considering all rejected this view and insisted that the curvature was not an outcome of oppositions but a harmonious entity, a manifestation of Irritability, being a motor-reaction following the perception of a stimulus at a sensitive region, the reaction being produced by the conduction of excitation from the perceptive to the motor region, whereby the uniform growth rate on all sides became rearranged as different growth rates on the two sides By Pfeffer it was discovered that, as long ago as 1824, Dutrochet had expressed the view that these curvatures were not forced on the plant by external conditions but self-generated at an indication from outside This view, which had been quite neglected, was now actively adopted.

The Darwins regarded these curvatures as essentially biological adaptive responses to external signals arising in the illuminational or gravitational state of the environment, the response and stimulus having become linked together by association during the evolution of plants, so producing forms more fit in the struggle for existence

In the preface to "The Power of Movement in Plants" we find Charles Darwin stressing his view that all these growth-curvatures of plants, which are so important as adaptations for existence, need not be regarded as a completely new type of growth-effect but may be derived as an extension of a pre-existing general tendency for growing plants to carry out continually circumnutatory bendings in all directions, even under uniform outside conditions. In later years, after criticism by Wiesner, Francis Darwin admitted that we still lack the knowledge to justify this view of the origin of growth-curvatures

We now pass on from our starting point to give an outline of the progress in collecting evidence about the nature of growth-curvatures from 1878 to 1908 when Darwin made his last contribution His twelve contributions are not referred to by title but are designated by roman numerals in order of publication and the date is given with each numeral The lectures and addresses are distinguished by dates only

Darwin's first contribution (I, 1880) would be the work presented in the "Movements in Plants" in association with his father Here we find recorded the early discovery of localisation of a special region sensitive to external directive stimuli The case was that of the localisation of the light-sensitive region in the tip of a seedling grass When a black glass tubular cap was fitted over this tip, one-sided light ceased to bring about the normal positive heliotropic curvature Experimental demonstration of a localised region for perception of gravity was more difficult because we have no materials that are opaque shields against gravitation Amputation of tips seemed then the only possible test after amputation of 1·5 mm. of the root tip no geotropic sensitivity was left and the root grew straight on, when laid horizontal If the root had been exposed horizontally before the tip was cut off, then curvature downwards would go on developing This seems satisfactory evidence of localisation of "gravi-perception," unless we admit that the shock of the operation had rendered the plant insensitive to gravity Owing to this unresolved doubt the decision remained in suspense for a period.

An early independent contribution by Darwin (III, 1882) with experiments on splitting the root-tip in various ways without actual amputation, provided some support for this localisation

A further contribution (IV, 1882) to the theory of curvatures was made by

Darwin with his research, conducted in Sachs' laboratory and published in Germany He examined the effect of uniform all-round light on the growth-rate of the root of White Mustard and found that light depressed this rate as compared with darkness Now it had already been shown that the Mustard root curves away from a source of light, implying a greater growth on the side towards the light This seemed to prove conclusively that the curvature could not be the direct outcome merely of the difference of illumination on the two sides. If the curve should have had this simple origin then the expectation would be more growth on the side away from the light and a positive heliotropic curvature *

In 1891 Darwin gave a presidential address to the Biology Section of the British Association, in which he stressed the evidence for regarding all curvatures as examples of irritability and showed how there had been a general abandonment of the view of De Candolle and a new adoption of the neglected early view of Dutrochet (1824) The second topic in this address was a survey of views upon the mechanical cause of the observed curvature, establishing that it was due to real differences of growth on the opposed sides

He also set out the criticism by Professor Wiesner (1881) who declined to believe that circumnutation of plants was universal and refused to regard it as the origin of heliotropism

In 1895 came a new discovery from Germany in which Pfeffer and Czapek took part They provided evidence that the perception of gravity really is located in the root-tip, by the use of Czapek's " glass boot " This boot is a very short piece of glass tube of a size to fit tightly on the root-tip, and bent at right angles in its middle, so that when once the root has grown into it the root-tip is kept at right angles to the general axis of the root If the root-axis is now placed horizontal with the booted tip pointing down, the gravity excitation does not arise and growth in a horizontal direction continues indefinitely The effects of other positions all support the same interpretation

Darwin's next contribution (VII, 1899) aimed at getting evidence of localisation of gravi-perception for the shoot as well as the root It was found possible to support very light grass seedlings by introducing their shoot-tips into glass tubes of exact fit If the glass tube were fixed horizontal then excitation of the tip would persist indefinitely It was found that seedlings thus supported carried out continuously curved growth so that a spiral or a knotted form of the shoot resulted

In 1901 Darwin gave the evening lecture to the British Association In this he set out the evidence obtained by the use of controlling glass tubes—the

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* It must be mentioned here that 33 years later it was pointed out by Blaauw that the side of the root away from the source of light is really more brightly lit *inside* owing to the root having the form of a cylindrical lens So the evidence of this experiment of Darwin's has now lost the force that had been universally attributed to it

work of Pfeffer and Czapek and his own experiments of 1899 Towards the end of his lecture he spoke of "unconscious memory" and the wide conception of memory formulated independently by Butler and by Hering, and expressed his inclination to interpret on these lines the acquisition of the power of curvature by plants in the course of evolution He would class animals and plants together from a psychological point of view

Just before this lecture was given, there came from the Continent a new conception the "statolith theory" brought forward in 1900 by Němec and by Haberlandt These physiologists and also Noll urged the view that the "falling starch grains" known to occur in root-tips and in the endodermis of stems are the intermediaries between the direction of gravity and the excitation of protoplasm With any change of position of the plant tissues these grains can be proved to fall to the lowest part of the cell that contains them and lie there pressing on the protoplasm at some quite unaccustomed region The cells containing falling starch are to be regarded then as sense-organs and the protoplasm of their upper, lower and radial walls must all have different excitabilities so that the appropriate movement is initiated to bring the cell into its normal position with the starch lying on the original bottom of the cell

To this theory of gravi-perception Darwin gave immediate adherence and carried out three short investigations which supported it. In contribution (IX, 1903), he showed that the stimulating effect of starch grains on an unaccustomed side of the cell is heightened if the tissue is kept in vibration on the prong of a tuning fork so that the starch grains dance on the protoplasm surface A later contribution (X, 1904) dealt with the exposure of roots and shoots to weak centrifugal force which had long been known to excite in the same way as gravity. He found that with a force sufficient to displace the starch grains the appropriate geotropic excitation occurs

In his next contribution (XI, 1904), he dealt with the problem of the similar occurrence of falling starch throughout a typical root system, made up of a primary root bearing secondary roots, which again bear tertiary roots Each of these three classes has its own type of directional reaction to gravity, the primary being positively geotropic, the secondaries diageotropic, while the tertiaries show no directional control by gravity. A significance was found for falling starch in the tips of the tertiary roots by their behaviour after amputation of the primary root At this juncture the secondaries take on the property of the primaries and the tertiaries behave diageotropically like secondaries. Without falling starch, it is held that this could not occur.

In 1904 Darwin was President of the Botanical Section of the British Association and gave an address which dealt very fully with all the recent work in support of the Statolith Theory. He dealt in addition especially with the problems of diageotropic organs and the phenomenon of rectipetality by which a freshly curved organ may recover its straight direction after the stimulus has ceased An early contribution on diageotropic leaves (II, 1881) comes up for mention in this relation

In 1906–7 Darwin gave a course of lectures in London University which were published in the "New Phytologist" There he expounded his satisfaction with the views of Semon who in his book "Die Mneme" (1904) provided a non-psychological nomenclature for dealing with vital behaviour of which the primary conception is the registration of stimuli as "engrams" in the organism These become associated with one another by repetition and can be called forth by one component of an original association of stimuli The association of heliotropic curvature with a light-stimulus would be an example of this

He also stressed the quantitative studies of the relations between stimulus and response initiated by Fitting in 1905 Fitting dealt with the effects of intensity and duration of stimuli, with the result of weak intermittent stimuli, and with the reaction to opposed stimuli, of equal or different duration, in serial alternation An early contribution by Darwin (V, 1888) dealt with the related problem of which angular position of a stem gives rise to maximal geotropic stimulus In 1908 Darwin was President of the British Association and delivered an inaugural address to the whole Association Addressing a general audience he presented the wider aspects of growth-curvatures as a problem of biology and adaptation He drew parallels between the behaviour of plants and that of Protozoa as studied by Jennings in his work of 1904 These were both presented as acquired habits, and changes of structural morphology in relation to external stimuli, as studied by Goebel, were brought into the same view of Habit He admitted in this address that he liked a blended mixture of psychology with physiology, and remarked that he generally got into hot water with the psychologists for this impropriety

Some of his scientific contributions not yet noted dealt with the subject of rhythm in plants and this found mention under the discussion of Habit The environment of the plant manifests a perpetual rhythm of day and night and the plant shows rhythmic habits in correspondence Many of these rhythms of movement persist for a time when constant light or constant dark is substituted for the normal alternation The "sleep movements" of leaves show this persistent rhythm At several dates in association with Miss Pertz, who co-operated in much of his research work in the Cambridge Botany School, he tried the production of rhythms, consisting of up and down geotropic curvatures, by exposing plants to opposed geotropic stimuli for alternate periods of 30 minutes. This subject was dealt with in contributions (VI, 1892) and (VIII 1903). In this treatment he used the "intermittent" klinostat designed by his brother Horace for this special work

His last contribution (XII, 1908) was a further critical study of the early subject of the geotropic curvature of seedling shoots fixed by their tips in a glass tube This was sent as a contribution for the Festschrift to Professor Wiesner of Vienna Professor Wiesner had as long ago as 1881 expressed his dissent from most of the conclusions about the significance of circumnutation set out in "The Movement of Plants," but he had combined his criticism with such courtesy and personal friendliness that the best human relations were always maintained And so Darwin's last contribution to this subject was sent to honour his earliest critic

The problems of biology endure for ever, and as an epilogue to the phase thus briefly pictured we should note the prologue to a new phase of the investigation of growth-movements which is now in full swing It is based on the discovery of "growth-substances" which can easily be isolated from plant-tissues These, applied locally, will accelerate the growth of one side of a shoot as against the other side with a smaller dose or none, thereby a typical growth-curvature results These actual substances are continually secreted by growing points and one-sided light or gravity alters the amount of them and more especially determines that they drift down more to the side destined for greater growth The connection between the "sensory region" and the "motor region" in plants is thus brought about by diffusing chemical hormones and not by any conduction of impulses analogous with nervous activity

II —DARWIN'S RESEARCHES ON THE CONTROL OF WATER-LOSS BY THE LIVING PLANT

The phenomenon of the control of loss of water by plants growing with their leaves in air which is subject to considerable variations of dryness, and intense variations of radiation has long interested philosophical biologists, who seek to make an orderly array of the complexity of physical, physiological and biological factors that are at work maintaining the survival of the individual plant

This field of work had a great attraction for Darwin, coming second only to the study of plant movement and having many aspects in common with it, as a problem of adaptation For the collection of data on water-loss he invented or developed a variety of special pieces of apparatus With each new one he re-surveyed the fundamental problems several times between the years 1897 and 1916 To give a chronological account of the seven papers published in that period would, then, involve a good deal of repetition. To avoid this the present notice of his work is a synthetic one in which the time-sequence is ignored and the whole body of results is rearranged under headings of the different major factors that affect water-loss As a preliminary to these divisions we present four sections, characterising the various sets of influences that have to be taken into account

The Physical Factors determining Water-Loss—Any damp object losing water to the surrounding air by evaporation may be presented formally as a physical system which has certain significant attributes, determining the rate of water-loss[1] The parts of this system, entitled the source, the sink and the diffusion path, are conceived as being located at three different positions in space We may characterise them as follows 1 The *source* where water is vaporised and the concentration of water-vapour, or humidity, E, is highest, in any steady state of the system. In a leaf this is located at the surface of the mesophyll cells 2 The *sink* where the vapour passes out of our field of enquiry This is located somewhere in the outer air Here the local humidity, *e*, will be least, the difference, E — *e*, being the prime determinant of rate of water-loss In natural conditions *e* is an independent meteorological variable Lying between these is (3) the *diffusion-path* of flow, constituted by the intercellular spaces and the stomatal pore The variable attribute of this part of the system is the cross-section of the path, which we may express as a resistance to diffusion, R, rising as the cross-section is diminished locally

Factors of General Physiology affecting Water-Loss—On the physical properties of our system, which is losing water, there are superposed certain vital properties general to all cells whether they are specially concerned with water-loss or not. One of these properties is the *local production of heat*, owing to respiratory metabolism This will accelerate vapour-production at the source and enable a supersaturation value of E to be maintained there, so that the cell can distil off water even with a saturated value of *e* at the sink This local production of heat will cause water-loss to be in excess of physical expectation, also, at all lower values of *e*

A second factor of general physiology arises from the increased *permeability of protoplasm* that is associated with light This altered state is known to affect metabolism and may also increase the vaporisation possibilities

Factors of Special Physiology Guard-Cells—These highly individualised cells in the epidermis of leaves have special functions in relation to water-loss As they are placed like throttle-valves round the openings of the intercellular diffusion-paths, they directly affect the transverse section of this path, and so the diffusion-resistance, R If they develop a large amount of osmotic substances they will withdraw water from the adjacent epidermis-cells and expand, thereby opening the stomatal pore The guard-cells may thus be said to compete with the epidermis for the available water. With low osmotic contents the guard-cells may lose water to the epidermis and become so flaccid that the pore is closed. The guard-cells appear then to be capable of exercising an independent initiative in the control of water-loss The manner of their working is to be explored later

Factor of Biological Control Stimulus to Guard-Cell Movement.—The most difficult problems for elucidation in this field of enquiry are those which arise when we consider the form-changes of the guard-cells as a special example of plant movement and then start to enquire precisely what changes in the environment act as stimuli to movement, and the nature of the chain of processes between the stimulus and the reaction. This reaction only takes place with living protoplasm, so the general state or "tonus" of protoplasm must affect the nature of the reaction

Such are the four strata of causal relations that interact on one another to determine the rate of water-loss of a leaf under the natural range of variation of the humidity and illumination of the leaf's environment. Though all the data could, in theory, be gathered merely by weighings, yet the interpretation of the variations of rate of loss that would be recorded involves a large part of the physiology of plants

Darwin worked through this field of phenomena at different times with different instruments, the principles of which we shall next describe and then pass to the results that he obtained

Darwin's Instruments for the Investigation of Water-Loss

Darwin had a striking natural talent for designing simple pieces of apparatus in response to his needs as an investigator A chronological series of those employed in his work on water-loss is set out below The potometer, the hygroscope and the porometer are so simple that they consist essentially of a principle clothed in the minimum of glass, rubber and cork The electric recorder was, of course, a highly complex scientific instrument, but this was designed by his brother, Horace Darwin primarily for non-biological purposes

The Potometer (1884) –This consisted of a glass reservoir of water into which a cut shoot was fixed air-tight so that it sucked out the water and created a reduced pressure in the apparatus Through a side capillary tube air bubbles are drawn in succession from outside to neutralise the reduced pressure The rate of the inflow of the air bubbles gives a measure of the rate of uptake of water by the shoot When steady conditions have once been established in the cut shoot, this "potometer-rate" gives an accurate measure of the loss of water by the surface of the shoot to the surrounding air Similar, but less sensitive, forms of this apparatus had been published by other physiologists

The Horn-Hygroscope (1897) —This little instrument is constructed out of small strips of horn-shaving specially treated so that the strip expands on the face exposed to moisture, it thus curls away from the surface of a leaf to a degree determined by the rate at which water vapour is pouring out from that surface. The strip is provided with an index bristle and a tiny cardboard scale and can be transferred from one leaf to another to give an empirical but quantitative measure of the water-loss, at any spot on the stomatal surface.

The Electric Recorder of Leaf-cooling (1904) —An evaporating leaf can be arranged so that, by contact with a platinum wire grid, it absorbs heat from this grid If the wire is part of the circuit of an electric-resistance-thermometer, the rise of the wire's resistance is a measure of its cooling and so a very sensitive recorder of the rate of evaporation from a leaf, minute by minute, can be constructed

The Porometer (1911) —This instrument was a new invention by Francis Darwin and differs from all the others in that it gives a measure of the degree of openness of the stomatal pore It provided a new power of analysis, as the change of total water-loss, which the other instruments alone record, can be examined as to whether it is associated with change of the aperture of the diffusion path (R) or is due only to alteration of the humidity of the source and the sink. The porometer in its simple form consists of a small glass bell-chamber which is sealed to the stomatal surface of a leaf by gentle pressure on a gelatine disc By sucking on a branched side-tube negative pressure can be established in the bell-chamber and a water column raised from an open water vessel The rate at which, when the suction is stopped, the sinking water column draws air through the stomata and intercellular spaces of the leaf gives a primary " rate of air flow " value which measures the grade of openness of the pores The square root of this value gives a measure of the diffusive capacity of the pore for the outward drift of water-vapour

The value of a measure of the rate of flow of air through stomata to give evidence of their state of openness was recognised by N J C Muller in 1873, but his procedure was too complicated for much scientific application

The Elimination of Stomatal Control (1914) —Darwin thought out a technique by which water-loss could be studied in a leaf from which the factor of control by guard-cells had been eliminated The preparation of such a leaf involves rather drastic treatment, but shoots of hardy evergreens seem to submit to this without serious injury The surfaces of the stem and leaves are rubbed over with vaseline so that all the stomatal apertures are blocked After this a definite number of razor cuts is made through the blade of each leaf so as to allow the escape of water vapour from the intercellular spaces to the outside sink by cut openings which are not throttled by guard-cells The number of cuts made is such as to give approximately the normal water-loss per leaf This water-loss is sometimes directly measured by weighing, but usually by attaching the shoot to a potometer for measuring water-intake as a guide to water-loss.

The Effect of Dryness and Humidity on the Water-Loss of Plants

Having now formalised the transpiring leaf as a physical system consisting of source plus diffusion path plus sink, and described the instruments with which the rate of water-loss has been investigated we pass to consider the actual behaviour of the living plant and the problem of how it comes to depart from that of a non-living system

In the present section we will deal with Darwin's work on the effects of changes in humidity, leaving the experimental work on illumination to the next section. For the integrated water-loss from a simple physical system we should start with the expectations that (1) decrease of humidity at the source would decrease water-loss, while (2) decrease of humidity at the sink would increase loss

In experimental work Darwin applied the first by cutting through the water channels in the leaf-stalk, and the second by moving the leaf still on the plant, from damp air to dry air

Reduction of Humidity at the Source —The effect on the water-loss of cutting or clamping the leaf-stalk can be observed with the horn hygroscope placed on the stomatal surface of the leaf, or recorded by placing the non-stomatal surface in close contact with the wire grid of the electric recorder Directly after cutting, either method shows a quite unexpected increase of water-loss which may get greater and greater for some 10 minutes, after which the water loss starts to decline and thenceforward goes on making a steady approach towards zero. There can be no physical explanation of the temporary rise of water-loss With the system in question, this could only be interpreted as due to wider opening of the stomatal pore. By the direct use of the porometer the opening was established, even to the extent that the diffusive potentiality of the stomata might be raised 1 8 times, immediately after cutting, and that this increase might last for 25 minutes before decrease sets in. To what was this strange opening of the pore to be attributed ? Cutting of leaf-stalks is no part of normal biology, but a clue to the nature of the mechanism involved might be revealed here Clearly any loss of water by the guard-cells should close them, but we observe an opening. Could it be due to deturgescence of the surrounding epidermis lowering the pressure with which it opposes the opening of individual guard-cells ?

This transitional increase of water-loss soon stops and there follows a rapid decline of water-loss, which is obvious and well-marked with all instruments It might have been possible that the decline was wholly due to falling humidity at the source, but Darwin's application of the porometer to withering leaves proved that the stomata shut progressively and rapidly so that the rate of water-loss is greatly reduced and thereby drying up is postponed What then causes the guard-cells to close in this second phase though they opened in the first phase ? By this time, the general water-loss of the leaf has proceeded further as its supplies are completely cut off, and the guard-cells themselves may be held to undergo loss of turgidity and mechanical collapse. Or possibly, as Darwin suggests, a stimulus may be transmitted to the guard-cells arising out of the general deturgescence that has taken place in the leaf

Variation of Humidity at the Sink.—We now pass from the unnatural condition examined in the previous section to consider the effect of alteration of the relative humidity of the surrounding air on the rate of the plant's water-loss. This may be said to be the most irregular natural variable in the biological experiences of plant life.

The physical expectation of the effect of reduced humidity at the external sink is, of course, increase of water-loss by reduction of e values, and thus the opposite of reduction of E, at the source. Within certain limits this expectation may be fulfilled and a steady increased water-loss be maintained in a lesser humidity provided this lesser humidity is itself still very high. In the region of experimentation that Darwin explored the variations of humidity used are more drastic and the more immediate effects of change are alone investigated. He found it was very rare to get a plant which did not react in the reverse of physical expectation when brought from a greenhouse into a dry laboratory, and thereby exhibit a reduction of water-loss. This reduction we should attribute to biological regulation by the reaction of the guard-cells. As we have indicated higher up, the behaviour when the humidity at the sink is lowered partly depends on the productive power of the source. Aloi had pointed out, and Darwin confirmed, that if the soil of a pot plant is abnormally saturated with water then a higher rate of water-loss initiated by a drier sink may be maintained without any regulatory closure of stomata. Here we should hold that no deturgescence took place.

In attempting to distinguish the part that is played by stomata, it is very important that we should acquire knowledge of the behaviour of the source when the superposed control of guard-cells is removed. This piece of analysis Darwin succeeded in carrying out by the waxing and cutting technique which has been already described (see p. xv). Treating leaves in this way, he arrived at the water-loss of the shoots by measuring their intake from a potometer. These experiments were carried out for a wide range of relative humidities of the external air, from 60 per cent. to 95 per cent.

Here in the absence of all guard-cell control the physical expectation is completely realised and the water-loss falls off in a straight line relation to the rising humidity at the sink. An array of data of this type had not been brought forward before and when set out graphically it was obvious that water-loss was not drifting towards a zero value in air of 100 per cent. relative humidity but that the slope indicated a quite considerable water-loss in saturated air, falling to zero only in a supersaturated state, located on the graph at about 105 per cent. relative humidity. Here than was a demonstration of the reality of the physiological expectation described on p. xiii that the continuous heat-production in respiration must give a "distillation effect."

Having acquired a picture of the behaviour of water-loss in the absence of guard-cells we may return to the normal leaf with its biological controls.

With normal plants in soil of normal humidity nearly all the observations that Darwin made with dry air indicate a special initial phase of increased water-loss followed by an adjusted phase in which the water-loss becomes markedly decreased The electrical recorder of the heat-absorption that is a concomitant of increased evaporation gives the clearest records of this initial effect Such an increase is in accord with physical expectation on reduction of e Whether there is an actual wider opening of stomata associated with it was not tested by the porometer, as the humidity of the air inside the porometer would require special controlling complications This initial phase may last perhaps 20 minutes. After 30 minutes or so there sets in a marked reduction of water-loss when the potted plant is brought from the greenhouse to the drier laboratory Numerous measurements with the horn-hygroscope prove the generality of this effect The reduction comes on before the eye detects any appearance of wilting in the leaf-blade It can only be attributed to closing of the stomatal aperture by deturgescence of the guard-cells The stimulus for the production of this biological control of water-loss Darwin would attribute to an initial general deturgescence of the leaf

We note that the initiation of this stomatal closure is less humidity at the sink, and yet it closely resembles the closure initiated by less humidity at the source As physical causes, these two lead to opposed effects upon rate of water-loss When we seek some common aspect to suggest as the directing cause of change of guard-cells, we find it in the state of reduced water content of the leaf tissues intervening between the leaf water-supply and the external air. The analogue of this on our physical system with water vapour drifting from E to e, the rate of flow being determined by $E - e$, is the fall of the mid-value $\frac{E+e}{2}$ that can be produced indifferently by lowering either E or e alone

It is in this general deturgescence rather than in direct deturgescence of guard-cells that Darwin sees the stimulus which brings about the reaction of the guard-cells. Since the date of Darwin's experimentation it has been shown by Thoday that leaves shrink a lot in area under conditions of strong water-loss such as exposure to direct sun This shrinkage is easily measured in a linear direction between Indian ink marks on the leaf surface and it might be that a series of such measurement would give the best direct index of the degree of openness of stomata

The Effect of Illumination and Darkness upon the Water-Loss of Plants

Taking up the factor of illumination we leave simple physical expectations behind us, as light has no primary effect upon the rate of evaporation from damp surfaces to air. We have seen, however, in our introduction that light might increase the efficiency of the sources of water-vapour (see p. xiii) We have now to bring together the experiments made by Darwin which seek out evidence of this general effect of light as well as such special effects of light on guard-cells as can be shown to take part in the biological control of water-loss As a foundation for this special enquiry we have the general view of the pioneer workers that the stomata on leaves are open by day and close at night

The first experimental finding that we have to record is the full confirmation of the reduction of water-loss that follows artificial darkening of leaves during the day-time Numerous observations with the potometer and the horn-hygroscope and a few with the electric recorder show that this effect is widely characteristic of typical leaves But this is an integrated effect and calls for analysis. The special waxing and cutting technique introduced by Darwin allowed him to examine the behaviour of the evaporating source apart from guard-cell control The measurements of intake by these shoots drawing water from a potometer showed higher values in the diffuse light of a north window than in darkness The ratios for the two states are very variable, ranging from no change to two-fold water-loss. Susceptibility seems to be seasonal as the summer increase is much greater The average ratio for a very large number of cases is 132/100 From these data one seems justified in holding that light-effects, of the nature of permeability changes, increase the evaporating efficiency of the mesophyll cells

By applying the porometer as a measure of factor R it was made quite clear that a considerable increase in the pore-opening is associated with light As light increases both of the factors making for increased water-loss, Darwin attempted the rather difficult task of distributing the total effect between the two factors The nature of the survey was to study how closely the variations of diffusion-potentiality, derived from the porometer rate of flow, follow the drift of total water traffic measured by the potometer The agreement is not very detailed, so that we cannot yet be sure of the correctness of the view that the influence of light on the guard-cells is by far the more important factor

It is interesting at this stage to point out a marked difference in the process of closing of stomata when brought about by darkening instead of by drought With darkening there is no sign whatever of the special transitional phase observed in drought in which, for some minutes, the stomata become more open. With darkening there is a slow steady decline of water-loss from the moment of darkening This is well seen in a few records with the electric recorder We conclude that light has only a single type of effect, a physiological acceleration of water-loss, while drought has two types of effects, physical in increasing loss and physiological in reducing loss

A more recent discovery in the physiology of guard-cells made since Darwin's work cannot be passed over without reference here. Iljin stressed the importance of the fact that the starch, which is so characteristic a content of guard-cells, readily passes over into sugar by a simple exposure to light thereby generating osmotic substance which will raise the water absorption and the turgor. On darkening the process is reversed. The light and darkness are held to work through the catalysts concerned in the starch-sugar conversion. Humidity of air has similar relations, exposure to dry air leads to starch-formation while humid air re-forms the osmotic sugar content Apparently we can now feel safe in interpolating this protoplasmic control of the amount of osmotic substance into the chain between the initiating dry air and the final change of form of the guard-cells, but the problem of how the initiatory process comes to affect the metabolic machinery of the guard-cells is still left on our hands

Nocturnal Closure of Stomata Day and Night Rhythm—The approach that was made by early workers to the influence of light upon stomata was to stress a phenomenon called the Nocturnal Closure of Stomata and to enquire about its mechanism, the range of its occurrence and its biological advantages Darwin surveyed this field with much thoroughness by the aid of his delicate indicator of relative water-loss—the horn-hygroscope—and the range of plants that the Cambridge Botanic Garden could provide Subsequent work with the porometer showed that it is really closure of stomata that reduces the water-loss at night It appears that closure begins well before sunset and reaches its maximum about 10 p m In the morning reopening begins before sunrise Darwin was much interested to enquire whether the constant repetitions of this rhythm of change had led to any inherent periodicity in the sequence of stomatal states As a test for this he used the formal method of finding out whether artificial light would bring on opening when the stomata are in normal nocturnal closure He found that the stomata are more readily induced to open by light applied at the end of the closure in early morning than by light applied during the closing period in the early evening The responsiveness of the cells is different at these two periods of time and thus evidence is provided of some inherent periodicity in the protoplasmic states. Darwin concluded that there is a definite inherent reaction of nocturnal closure and this has been more clearly brought out in experiments since his date Maskell has observed that powerful constant artificial light all through the 24 hours will not prevent the stomata of a leaf closing during the early evening to a minimal opening at 10 p m It is interesting to find that in this constant strong light they do not remain shut, but soon start to reopen and may be fully open again long before the normal time

Darwin speculated not very successfully about the biological advantage of nocturnal closure, although he found that aquatic and marsh plants hardly show it and it is only slight in leaves which have a special "sleep" position of their leaves at night

III.—DARWIN'S OTHER BIOLOGICAL WORKS

Omitting any reference to the contents of the large number of short papers that Darwin published, there are certain of his scientific activities that led to the production of books and so call for mention here

His well-known class-book " The Practical Physiology of Plants " appeared in 1894. This book was built up on his experience in holding a practical class in this subject for many years No book of this type had been available in English before, as Sachs' "Experimentalphysiologie" of 1865 had never been translated Darwin's book ran into several editions

He also published a small book entitled "Elements of Botany" (1895) which presented the lectures that he had given in Elementary Biology to medical students.

The " Makers of British Botany " (1913) was a volume of composite authorship to which Darwin contributed an article on Stephen Hales who, though not a botanist, created, all by himself in the exercise of his power as an investigator, many philosophical aspects of plant physiology.

Darwin's edition (1903) of " A Naturalist's Calendar " was referred to in the biographical part of this notice

To end this list we may mention that Darwin lectured for a number of years on the Natural History of Plants Finally he printed his lecture-notes with descriptions of all the plants to be examined in the practical work Sets of sheets amounting to 100 folio pages were bound up for private circulation, but never published

F F. B

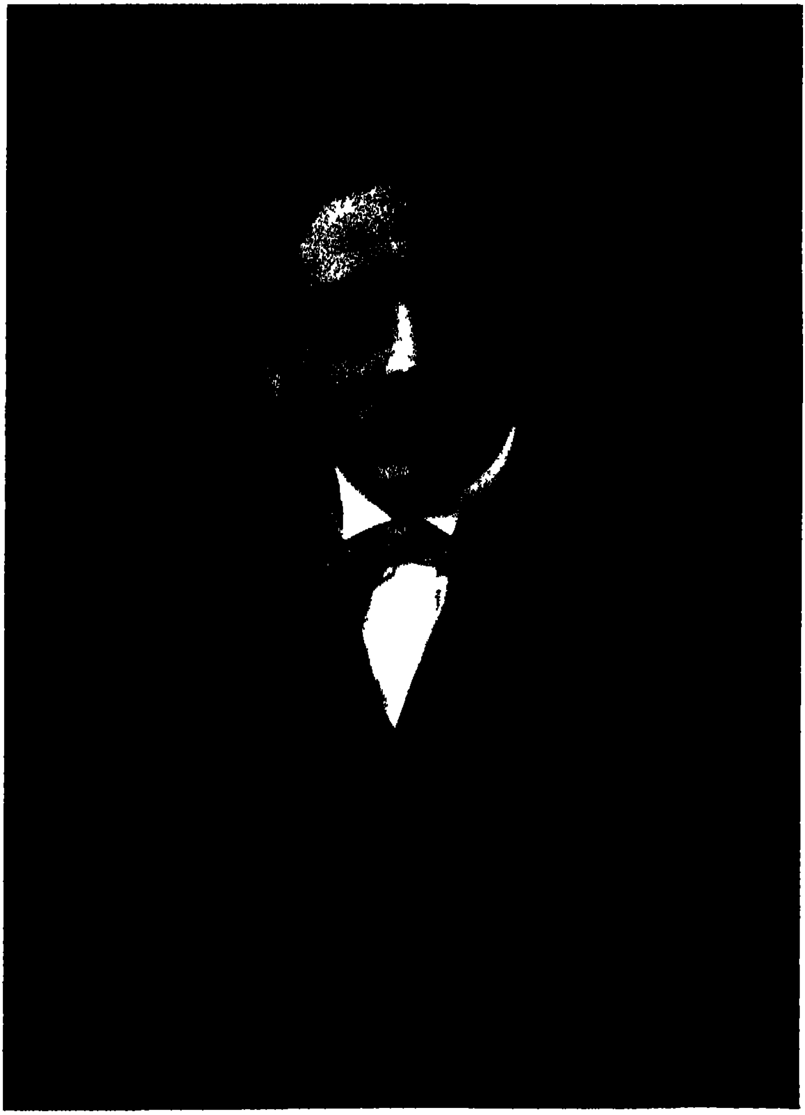
SAMUEL BARNETT SCHRYVER—1869–1929.

Samuel Barnett Schryver, who died on August 21, 1929, was the son of the late Lewis and Elisabeth Schryver and was born in London on March 15, 1869. He was educated at University College School and University College, London After taking his degree of Doctor of Science at London University he worked for a time at the University of Leipzig where he took a Ph D degree. After his return to England he held an appointment from 1893–97 as assistant lecturer and demonstrator of chemistry at the University College, Liverpool From 1898 to 1901 he was on the staff of the Wellcome Research Laboratories, leaving that position to become Lecturer in Physiological Chemistry at University College, London This post he relinquished in 1907 on his appointment as Physiological Chemist to the Research Institute of the Cancer Hospital. In 1913 he was appointed Assistant Professor of Biochemistry at the Imperial College of Science and Technology, becoming full Professor in 1920 He was elected to the Fellowship of the Royal Society in 1928

Schryver's earlier work was in pure chemistry, *e g*, on the quarternary ammonium compounds, the oxidation products of turpentine, the structure of morphine, etc On his appointment at University College his attention was naturally directed to biochemical problems and it was this field of chemistry which engaged his interest until his death. His earlier work in this field, partly in collaboration with Dr Lane Claypon, was on the phenomenon of autolysis, he also made observations on the excretion of nitrogen by normal individuals While at University College he published a report on Food and Food Products for the Local Government Board In the course of this work he markedly improved Rimini's test for formaldehyde so that it would detect 1 part in 1,000,000 This improved method, which is often known by his name, he later applied to the detection of formaldehyde produced when crude chlorophyll is exposed to sunlight

While he was on the staff of the Cancer Hospital he worked, with Dr. C. Singer, on the gastric juice in malignant and non-malignant disease of the stomach and duodenum Schryver was interested in the state of aggregation of organic substances and this led him to the study of clot formation and especially of the clotting of milk by rennin He also studied the unconjugated acids of ox bile and devised a method for the separation of cholalic, choleic and deoxycholeic acids.

His research work at the Imperial College followed two main directions. There was on the one hand his work on plants, which consisted of a study of the proteins of leaves and of the constituents of the cell-wall. The work on the leaf proteins was begun with the co-operation of Dr A. C. Chibnall, who



has been responsible for its later developments. While engaged on work on the cell-wall he investigated pectins and hemicelluloses. He was led to the view that both pectic acid and the hemicelluloses were complexes of uronic acid with sugars, for which complexes he suggested the name polyuronides. The other main direction of his work was the result of investigations undertaken for the Adhesives Committee which was appointed in 1917 and, later (1919), became the Adhesives Research Committee of the Department of Scientific and Industrial Research. These investigations led him to an elaborate study of the purification and properties of gelatin and, as a corollary to this, to detailed work on the separation of the products of protein hydrolysis. In his work on gelatin he studied the formation of gelatin from its precursor in which it was shown that the composition of gelatin was a variable one depending on the previous treatment of the precursor or of the gelatin itself. Intensive studies were undertaken on the purification of gelatin in which electrodialysis was found to be very effective, though owing to the action of water such purified material was apparently always contaminated by the products of the action of the water, which produced even at a low temperature a change, irreversible in nature, which could be observed in a few hours

His most striking work—undertaken with his students—was that on the separation of the products of protein hydrolysis, in which he developed Siegfried's barium carbamate method for the separation of amino-acids. By its use he was able to isolate several hitherto unknown substances. From isinglass and edestin he obtained hydroxylysine, and from the glutelin of oats two new aminohydroxy-acids, aminohydroxybutyric and aminohydroxyvaleric acid, the first forming an insoluble and the second a soluble copper salt. From oat and from the protein of the castor oil bean he obtained an eight-carbon acid, $C_8H_{15}O_3N_3$, which he termed protoctin. From gelatin which had been treated with acid he separated *dl*-lysine.

From 1918 onwards he was also occupied with investigations on the nutrition and autolysis of yeast, in the course of which the interesting observation was made that washed yeast autolyses much more slowly than unwashed.

He published two books, one in 1909 on "The General Characters of the Proteins," and "An Introduction to the Study of Biological Chemistry" which appeared in 1918. His scientific papers and notes number nearly a hundred.

He married in 1910 Elsie, one of the two daughters of Arthur Davis, a well-known Hebrew scholar. He leaves two daughters.

Schryver was an enthusiastic investigator, fertile in ideas and with a marked originality of outlook. His research students found him not only an inspiring teacher but one who took the keenest interest in their welfare and was always ready with advice and generous help He was a man of wide interests, a lover of music with an appreciation of modern composers In his writings he combined clarity of thought with a feeling for *le mot juste*. The conclusion of his scientific career at a comparatively early age, while he was still in the full tide of investigation and the leader of an active school of research, was a great loss to biochemistry

Acknowledgment is made to the writer of the obituary notice in the 'Journal of the Chemical Society'

V H B

WILLIAM CARMICHAEL M'INTOSH—1838-1931

At the time of his death Professor M'Intosh was one of the senior fellows of the Royal Society, having been elected so long ago as 1877, and he had outlived nearly all the associates of his prime To the younger zoologists of the present day, perhaps, " the man was no more than a voice, In the white winter of his age, to those With whom he dwelt, new faces, other minds," yet to any who cared for the historical development of their science it was something memorable to talk to a man who had known Edward Forbes and sat at the feet of John Goodsir, and who, having met Monro *tertius*, lived to discuss the Polychæta with Monro's great grandson

William Carmichael M'Intosh was born in St Andrews on October 10, 1838 His father, Baillie John M'Intosh, was a builder and contractor in St Andrews and, as town councillor and magistrate, was prominent for many years in the civic life of that ancient city. It was probably his mother who awakened the boy's interest in the living things of the sea, for, as he tells us, it was she who, with his sisters, long afterwards kept him supplied with material for his researches when he was living inland at Murthly One of the sisters, Roberta, afterwards the wife of Dr Albert Günther, was an accomplished artist, and her exquisite coloured drawings adorn many of her brother's works. It must have been in his boyhood also that he met Edward Forbes and accompanied him on seashore excursions at St. Andrews

After his school-days at Madras College, M'Intosh entered the University of St. Andrews (1853-1857) and later proceeded to Edinburgh where he studied medicine, taking the diplomas of medicine and surgery and receiving the degree of M.D. in 1860 On graduating, he was appointed Assistant Physician to Murray's Royal Asylum, Perth, and specialised in the study of mental disease, on which subject he published a number of papers. In 1863 he became Medical Superintendent of Perth District Asylum at Murthly, a position which he held until 1882 In later life, when occupied with widely different work, he retained his interest in his old profession, and in 1890, as president of the

McIntosh

Fifeshire Medical Association, he gave an address on the surgical cases that had come under his notice in the twenty years of asylum practice at Murthly.

Through all these years, however, the interests of his boyhood continued to claim an increasing share of his attention. At Edinburgh, where he studied anatomy under John Goodsir, he had published a paper on the arrangement of the muscle-fibres in the bird's heart. His thesis for the degree of M.D., for which he received a gold medal, was entitled "Observations and Experiments on *Carcinus mænas*" and dealt mainly with the physiology of the nervous system in the shore-crab and its reactions to various drugs It seems to have been the influence of another of his teachers, G. J Allman, that turned his attention decisively to marine zoology. He accompanied Allman on dredging excursions in the Firth of Forth and learned from him the use of the dredge and the tow-net. He began early the long series of faunistic papers which were continued down to the last years of his life. Much of his material, as already mentioned, came from St. Andrews, but he also made excursions as far afield as the Channel Islands, the Shetlands, and the Outer Hebrides to study the marine animals A series of papers on the St Andrews fauna was afterwards reprinted in a volume, "The Marine Invertebrates and Fishes of St. Andrews," published in 1875

The groups to which M'Intosh's attention was specially given were the Nemertine and Polychæte Worms. A series of papers on these led up to his great "Monograph of the British Marine Annelids," published by the Ray Society, of which the first two parts, dealing with the Nemertinea, appeared in 1873 and 1874

M'Intosh's work on the Nemertinea was the most important of his contributions to pure zoology. The worms of this group are in some ways peculiarly difficult to study. Many of them are too large for microscopic examination of the entire animals to reveal much of their internal structure Their pulpy consistency and the fact that all the organs are embedded in a continuous parenchyma make dissection almost impossible, and the technique of section-cutting was at that time in its infancy Nevertheless, M'Intosh succeeded, by methods that now seem primitive, in making out the main features of their anatomy. Later investigators, working with microtome and embedding-bath, have added many details but have found surprisingly little to correct M'Intosh failed to discover the fine canals that form the excretory system, but, on the other hand, he was the first to make clear the relations of the characteristic and complicated proboscis and its sheath. Dr. Otto Bürger in his monograph of the Naples Nemertinea (1895) pays cordial tribute to "Die glänzende Monographie der Britischen Nemertinen von M'Intosh, die in faunistischer und systematischer Hinsicht Unschätzbares geleistet hat."

After the publication of the first two parts the Annelid monograph was laid aside and it was not until 1900 that a beginning was made with the Polychæta.

Thereafter, the successive parts appeared at intervals of some years until in 1923 the present writer had the unusual experience of seeing through the press the final sheets of a work that had begun publication when he was in his cradle, half a century before The monograph is illustrated by numerous exquisitely executed plates, many of them in colour, largely from drawings by Mrs Gunther. Although the work will always remain one of the classics of the subject, it inevitably suffered from the long period occupied in its publication M Intosh spared no pains in keeping abreast of the most recent literature, but it was not to be expected that a work planned in 1873 should reflect adequately the point of view of 1923

Another important work on the Polychæta was the report on the collection obtained by the "Challenger" Expedition, which appeared in two large quarto volumes in 1885 Its preparation occupied M'Intosh for seven years Among many other novelties described in it was the remarkable *Syllis ramosa*, which still remains the only known instance of lateral budding in a normal Polychæte

While engaged on this collection, M Intosh received from the "Challenger" office specimens of various problematical organisms which had been rejected by other specialists as not belonging to the groups they were studying Among these was perhaps the greatest zoological novelty obtained by the expedition, the animal to which he gave the name *Cephalodiscus*. He gave a careful and detailed description of this and correctly recognised its affinity with Allman's *Rhabdopleura*. It was Harmer, however who in a short but pregnant appendix to M'Intosh's report, pointed out the relationship of *Cephalodiscus* to *Balanoglossus* and the Chordata

Meanwhile events had occurred that diverted M'Intosh's activities into new channels In 1882 three of the chairs of Natural History in the Scottish Universities received new occupants Sir Wyville Thomson died and was succeeded at Edinburgh by Cossar Ewart, then professor at Aberdeen The Aberdeen chair was filled by the translation of Alleyne Nicholson from St Andrews, and M'Intosh returned to his native city as Professor of Natural History In his inaugural address, after giving a sketch of the advances in marine zoology and particularly of the deep-sea explorations of the preceding decade, he strongly urged the establishment of a marine laboratory at St Andrews Although the plans which he drew up for this could not be realized at the time, an opportunity came a few years later

In 1883 a Royal Commission was appointed under the chairmanship of Lord Dalhousie, with Huxley as scientific member, to inquire into the problems raised by the introduction of beam-trawling and its effect upon the sea-fisheries of Scotland M'Intosh, who had published several papers on the life-history of the salmon while he was at Murthly, was appointed to conduct scientific investigations for the Commission. He threw himself into the task with his wonted energy and enthusiasm, and, although he was by no means immune to the discomforts of work at sea, carried out a number of experimental cruises on board a commercial trawler and produced a report within a year. Perceiving the need for laboratory work on shore, he succeeded, with the aid of a small grant from the Commission, in establishing at St Andrews the first marine laboratory in Great Britain This was opened in 1884 in a small wooden building which had been hastily erected a few years before for use as a fever hospital. The building was barely weather-tight, the only boat was a small yawl, and the staff consisted of a single fisherman, known to generations of students as "Professor" Brown It is not too much to say that for the next ten years at least this modest establishment was the headquarters of British marine research Besides M'Intosh and his pupils the list of zoologists who worked there includes the names of Ray Lankester, A G. Bourne, Hubrecht, Nansen, Burdon Sanderson, Gotch, and many others of like distinction When, in 1896, the wooden laboratory was replaced by the stone building provided by the generosity of Dr C H Gatty, the example of St Andrews had been followed elsewhere and the necessary support became increasingly hard to obtain Since the war, the Gatty Laboratory has stood shuttered and deserted

It was in 1865 that the late Professor G O Sars, in Norway, laid the foundation of scientific fishery research in a little paper in which he traced the life-history of the cod and showed that its eggs float at the surface of the sea His work was followed up to some extent, especially in America, but it was M'Intosh and his pupils who first showed that all our marine food-fishes, with the notable exception of the Herring, have pelagic floating eggs, and by their detailed descriptions of the eggs and larval stages made possible the modern developments of marine fishery research. M'Intosh's work in this field was summarized in a book "Life Histories of British Marine Food-Fishes" which he wrote together with Dr A. T Masterman and published in 1897 His views on more general fishery questions were set forth in another work "The Resources of the Sea," published in 1898, and in a second edition in 1921 This book deals with many questions that are still matters of controversy, but its main conclusion will meet with a considerable measure of assent—"It is true that the larger examples of the common species of food-fishes become fewer by persistent fishing, but it cannot be said that, in the case of either round or flat fishes in the majority of the areas, signs of extinction are apparent"

As a zoologist, M'Intosh's interests were those of the field naturalist, and it was perhaps unfortunate that the main part of his working life fell in a period when zoologists tended to be interested only in the problems of morphology and embryology. It is evident from his writings, and it was still more evident to those who came in contact with him, that he had a clearer conception of the broad outlines of marine ecology than many of his contemporaries. The late

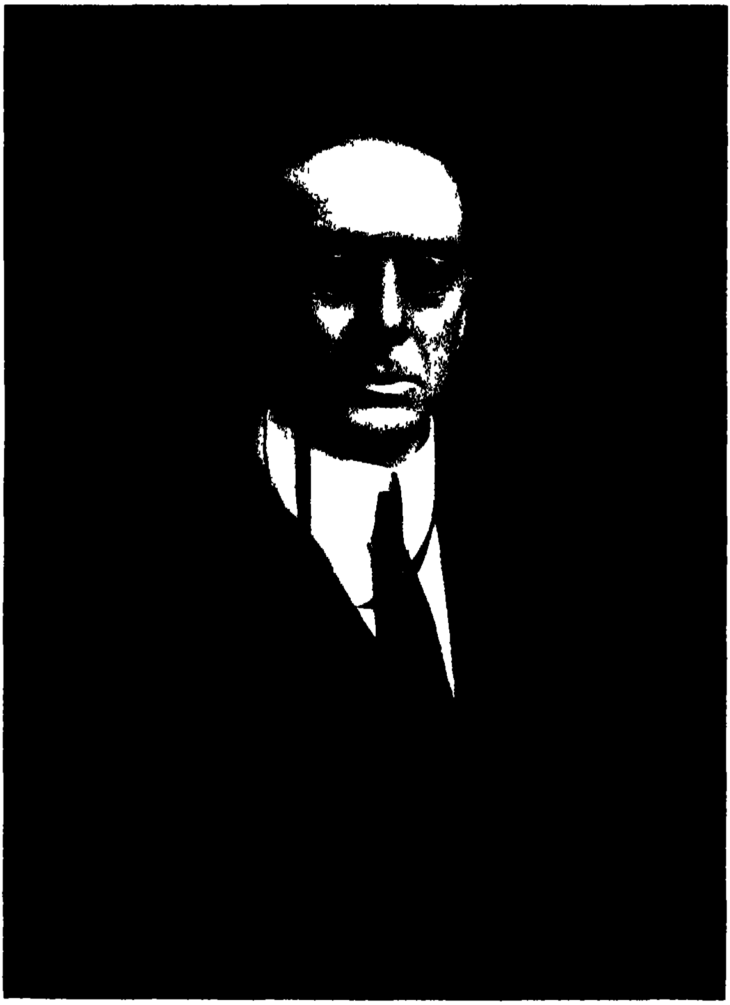
Dr. Nelson Annandale once told the present writer that his first realization of what ecology meant came from a seaside walk with M'Intosh.

No good highlander really dislikes a fight, and at the council table or in public controversy M'Intosh was accustomed to maintain his point of view with a tenacity which his opponents were apt to describe as obstinacy. In academic politics he played a conspicuous part, as, for instance, in the embittered disputes that delayed the incorporation of University College, Dundee, in the University of St Andrews

Many academic and other honours came to M'Intosh in the course of his long life He was a recipient of the Royal Medal in 1899, and in 1924 he was awarded the Linnean medal by the Linnean Society. It can be mentioned now that the first suggestion of his name for the latter honour came from his life-long friend Sir Ray Lankester Of the many scientific societies of which he was a member, none claimed more of his interest in his later years than the Ray Society, which he had joined so long ago as 1863 He became its president in 1913 and thenceforward took a very active part in the management of its affairs. In spite of his remoteness from London he rarely missed a council meeting, and it was in returning from such a meeting in March that a bitter east wind and the inconsiderateness of some fellow-passengers led to a chill that even his iron constitution failed to shake off. The first of April saw the close of a career that was pursued to the very end with single-minded devotion and an industry that seemed as if it could never flag

The portrait is reproduced from a photograph kindly lent by M'Intosh's nephew, Dr. R. T. Günther. It was taken in August, 1928, by Miss Alice Mitchell

W. T. C.



Walter E. Dixon

WALTER ERNEST DIXON—1871-1931.

Walter Ernest Dixon, who died suddenly on August 16 last year, was one of the small number of whole-time teachers of pharmacology in this country. His premature death at the age of 60 has deprived his colleagues of a charming and much-loved friend, and is moreover an irreparable loss to his subject, for not only had he done pioneer work in establishing the science, but also he had a remarkable gift for interpreting to the medical profession and even to the general public the meaning and importance of the scientific study of the action of drugs

Dixon was educated at Dulwich and at St Thomas's Hospital and qualified in 1895 After holding house appointments he took a post in the physiological department and henceforward devoted himself to teaching and research

He published his first scientific work whilst holding a Salter's scholarship at St. Thomas's Hospital These papers were on the two drugs of addiction, mescal and cannabis indica, and on the action of orchitic extracts The two subjects of drug addiction and endocrinology continued to interest him throughout his life In 1899 he became assistant to the Downing Professor of Medicine and resided at or near Cambridge for the remainder of his life For a time he held the chair of Pharmacology at King's College, London, concurrently with this appointment, but in 1919 he resigned the chair on his appointment to a readership at Cambridge He was elected a Fellow of the Royal Society in 1911. During the years 1914–1919 he served in the Royal Navy, and was employed on important services abroad, for which he was awarded the O B.E at the termination of the war

It is difficult to give any short account of Dixon's scientific work, because of its wide variety He had an exuberance of ideas and new subjects interested him intensely He did pioneer work in opening up numerous new fields of research, but often left their full exploitation to others

In collaboration with Brodie he investigated the innervation of the pulmonary vessels and the bronchial muscles This work which was published in 1903 and 1904 led, amongst other results, to the conclusion that spasm of the bronchial muscles was the fundamental cause of acute attacks of asthma.

In collaboration with Halliburton he investigated the factors controlling the secretion of cerebro-spinal fluid, and the question of absorption of substances from this fluid. This work was published from 1912 to 1914

One of the most important pieces of work that he did in post-war years was the demonstration of the influence of the ovary on the pituitary gland. In 1923 he found that the posterior lobe of this gland, secreting into the cerebrospinal fluid, was specifically excited to this secretion by ovarian extracts.

Next year, with F. H. A. Marshall, he showed that this effect was only produced by extracts made from ovaries which did not contain corpora lutea This was some of the first work to reveal a portion of the complex mechanisms relating the activities of the pituitary gland and the ovaries.

In addition to the researches mentioned, Dixon published a large number of papers describing the action of individual drugs, and also contributed many articles to systems of pharmacology. Any summary of Dixon's publications would, however, fail to indicate his influence on the science of pharmacology. In the first place he was a brilliant and stimulating lecturer, and as Dr H. H Dale has remarked "Pharmacological teaching in England, when Dixon began, presented an unappetising mixture of half obsolete *materia medica* with empirical therapeutics. In Dixon's hands it became a lively adventure in experimental science." A whole generation of students of medicine at Cambridge was taught by him, and this direct personal influence was extended to a wider field by his excellent Manual of Pharmacology.

Furthermore Dixon had a great influence in promoting research His enthusiasm for new ideas has already been mentioned, but it must be added that he was true to his chosen motto "*Dire n'est rien faire est tout,*" and showed great enterprise in devising new experimental methods He had, moreover, a remarkable instinct for recognising promising new lines of research, for example, as early as 1906 he produced experimental evidence for the hypothesis that the effect of impulses in the vagus nerve was transmitted by liberation of a chemical substance at the peripheral terminations of the nerve, although it was fifteen years later before conclusive evidence for this view was supplied by other workers

In post-war years much of Dixon's time was occupied with public work He served on international committees of experts that advised the League of Nations on drug addiction, and on the biological standardisation of drugs He also served on many Government committees which dealt with such subjects as food preservatives, lead-ethyl and drug addiction. He also was an active member of the British Medical Association, and did much to guide its scientific activities. Amongst others of his activities he was English editor of the 'Journal of Pharmacology and Experimental Therapeutics,' and also edited the pharmacological sections of the British Pharmaceutical Codex

Dixon always felt that the science of pharmacology was dangerously neglected in this country, that our parasitic position as regards the discovery of new drugs was a national reproach, and that in the teaching of medicine the subject of treatment suffered undue neglect. He worked indefatigably to advance his subject, and the importance of his efforts will be more than ever recognised now that they have ceased.

During recent years he received many honours. He was president of the Physiological Section of the British Association in 1929, and in 1930 he was elected a Fellow of the Royal College of Physicians and received the honorary

J. A. Towle

degree of LL.D. from the University of Manitoba. He had recently been appointed Croonian lecturer at the Royal College of Physicians of London.

The great influence that he exerted was largely due to his power of inspiring affection. His was a warm-hearted and unselfish personality, full of interest and charm. Students who worked under him found not only an inspiring teacher but also a kindly friend always ready to give helpful and shrewd advice

Dixon's home life was closely linked to his scientific activities, for he and his wife were the most charming of hosts, and for a generation made their home a centre of hospitality to his colleagues from this and other countries.

A J. C.

FRANCIS ALEXANDER TOWLE—1874–1932

Assistant Secretary to the Royal Society, 1921–1932

By the death of Mr Francis Towle, on January 10 of this year, the Royal Society lost one who had given faithful and efficient service to its affairs for over thirty years It seems fitting that this obituary series, though usually limited to our Fellows, should include a brief memorial notice of one who was still serving the Society as its chief permanent official at the time of his death, and was wholly devoted to its interests even in his last illness

Mr. Towle was born at Nottingham on March 19, 1874, but his parents moved to Birmingham two years later, and he was educated at King Edward's School in that city, and subsequently at the Midland Institute Municipal Technical School As a youth of seventeen years he came to London, having obtained a post with the Art Union of London, a firm engaged in the reproduction of works of art by photo-engraving To this early employment he probably owed a skill and interest in photography which he retained for many years, becoming a well-known member of the Camera Club In 1899 he joined the office staff of the Royal Society as Assistant Clerk to the Government Grant Committee, and was promoted to the Clerkship two years later. He held this position for twenty years, during which he made friendly contact with many Fellows, earning their regard by his courteous help, and becoming devoted to the history and the traditions of the Society which he served. When the post of Assistant Secretary and Librarian became vacant in 1921, the Council promoted Mr. Towle to this chief position on the permanent staff, and their decision was generally welcomed by the Fellows.

Mr. Towle indeed was fitted for the position by many qualities of character and temperament. He was conscientious, modest and entirely unselfish;

he had a quiet and genial manner, and the gift of a gentle, though on occasion incisive, humour He was unmarried, and his family and personal ties were so few that it was a matter for comment among the Fellows who knew him best how little they knew of his life apart from the Society His life, indeed, became increasingly there centred, and his personal relationships were largely those which his official position brought His appreciation of the Society's scientific activities was not, at any point, more than that of a thoughtful layman His nearest approaches to directly scientific interests were through photography and practical engineering, both practised as hobbies He made no pretension even to expert knowledge of the Society's wealth of early scientific documents He had the modest kind of ability which finds its scope in the efficient oversight of practical detail and routine He was a staunch champion of the Society's interests, enthusiastically loyal to its traditions, jealous for its prestige, and quick to suspect encroachment on its prerogative If you ventured to criticise the Society, you did so at the risk of his enmity

Such absorption in the life of an ancient Society could hardly fail to induce a habit of respect for precedent, and of suspicion with regard to change It might well be argued, indeed, that a proper part of the function of an Assistant Secretary, with a life-long experience of the Society, is to maintain and to transmit its traditional usages, and to protect them from unnecessary or inadvertent change, at the hands of Fellows holding office for relatively short terms Mr Towle was still a comparatively young man at the time of his death, but this conservative tendency was already manifest in his attitude to matters of form and detail On many occasions I have heard him lament the lapse of our Fellows, especially of those more recently elected, from the standard of formal courtesy thought proper to communications with the Society in the Victorian era, towards the end of which he entered its service He never quite reconciled himself to the custom of smoking in the tea-room before the Society's meetings, and his sense of propriety was seriously offended by the casual dress in which some younger investigators attended to communicate their work at the Society's invitation.

While he was thus tenacious of tradition in matters of form, Mr Towle moved loyally with the Society in the great change which occurred in its activities in the period following the War At the time of his appointment, in 1921, as Assistant Secretary, the Society was just entering upon a period of peculiar difficulty The first two of a series of major gifts and bequests, which have in recent years been entrusted to the Society to be administered for the support of research, had then been received In the years that followed the Society's responsibilities for the administration of such Trusts continued to expand, while the great rise in costs had reduced the value of its income, for its own ordinary purposes, to the point of serious embarrassment The annual subscriptions of the Fellows were increased; but the new and progressive demands could not have been met without Mr Towle's loyal acceptance of the increasing volume of work for himself and his staff, and his watchful care for economy in the Society's own expenditure. When the situation recently became easier, partly through generous bequests for the Society's general purposes, none was more eager than he for improvements, many long overdue, in the amenities, the decoration and the lighting of the Society's rooms, and in the care of its rich heritage of fine portraits

Most of the Fellows knew Mr. Towle as a courteous and obliging official, some knew him on a more intimate footing of pleasant friendship, but only those who have been responsible for the administration of the Society's business know its full debt to the service of one whose life was quietly but completely devoted to its interests

H. H D.

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